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(54) Title: TRIPEPTIDES HAVING A HYDROXYPROLINE ETHER OF A SUBSTITUTED QUINOLINE FOR THE INHIBITION OF NS3 (HEPATITIS C)
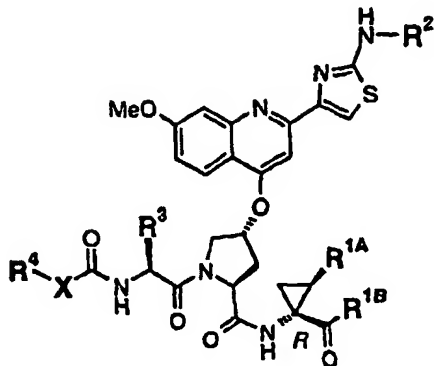
(I)

(57) Abstract: Disclosed herein are compounds of formula (I), wherein R^1 is hydroxy or $NHSO_2R^{1A}$ wherein R^{1A} is (C_{1-8})alkyl, (C_{3-7})cycloalkyl or {(C_{1-6})alkyl-(C_{3-7})cycloalkyl}, which are all optionally substituted from 1 to 3 times with halo, cyano, nitro, O-(C_{1-6})alkyl, amido, amino or phenyl, or R^{1A} is C_6 or C_{10} aryl which is optionally substituted from 1 to 3 times with halo, cyano, nitro, (C_{1-6})alkyl, O-(C_{1-6})alkyl, amido, amino or phenyl; R^2 is (C_{4-6})cycloalkyl; R^3 is t-btuyl or (C_{5-6})cycloalkyl and R^4 is (C_{4-6})cycloalkyl; or a pharmaceutically acceptable salt thereof. The compounds are useful as inhibitors of HCV NS3 protease for the treatment of Hepatitis C.

TRIPEPTIDES HAVING A HYDROXYPROLINE ETHER OF A SUBSTITUTED QUINOLINE FOR THE
INHIBITION OF NS3 (HEPATITIS C)

FIELD OF THE INVENTION

The present invention relates to compounds, processes for their synthesis,

5   compositions and methods for the treatment of hepatitis C virus (HCV) infection. In
particular, the present invention provides novel peptide analogs, pharmaceutical
compositions containing such analogs and methods for using these analogs in the
treatment of HCV infection.

BACKGROUND OF THE INVENTION

10   Hepatitis C virus (HCV) is the major etiological agent of post-transfusion and
community-acquired non-A non-B hepatitis worldwide. It is estimated that over 200
million people worldwide are infected by the virus. A high percentage of carriers
become chronically infected and many progress to chronic liver disease, so-called

15   chronic hepatitis C. This group is in turn at high risk for serious liver disease such
as liver cirrhosis, hepatocellular carcinoma and terminal liver disease leading to
death.

The mechanism by which HCV establishes viral persistence and causes a high rate

20   of chronic liver disease has not been thoroughly elucidated. It is not known how
HCV interacts with and evades the host immune system. In addition, the roles of
cellular and humoral immune responses in protection against HCV infection and
disease have yet to be established. Immunoglobulins have been reported for
prophylaxis of transfusion-associated viral hepatitis, however, the Center for

25   Disease Control does not presently recommend immunoglobulins treatment for this
purpose. The lack of an effective protective immune response is hampering the
development of a vaccine or adequate post-exposure prophylaxis measures, so in
the near-term, hopes are firmly pinned on antiviral interventions.

30   Various clinical studies have been conducted with the goal of identifying
pharmaceutical agents capable of effectively treating HCV infection in patients
afflicted with chronic hepatitis C. These studies have involved the use of interferon-
alpha, alone and in combination with other antiviral agents. Such studies have
shown that a substantial number of the participants do not respond to these

therapies, and of those that do respond favorably, a large proportion were found to relapse after termination of treatment.

5   Until recently, interferon (IFN) was the only available therapy of proven benefit approved in the clinic for patients with chronic hepatitis C. However the sustained response rate is low, and interferon treatment also induces severe side-effects (i.e. retinopathy, thyroiditis, acute pancreatitis, depression) that diminish the quality of life of treated patients. Recently, interferon in combination with ribavirin has been approved for patients non-responsive to IFN alone. However, the side effects

10   caused by IFN are not alleviated with this combination therapy. Pegylated forms of interferons such as PEG-Intron® and Pegasys® can apparently partially address these deleterious side-effects but antiviral drugs still remain the avenue of choice for oral treatment of HCV.

15   Therefore, a need exists for the development of effective antiviral agents for treatment of HCV infection that overcome the limitations of existing pharmaceutical therapies.

        HCV is an enveloped positive strand RNA virus in the Flaviviridae family. The single

20   strand HCV RNA genome is approximately 9500 nucleotides in length and has a single open reading frame (ORF) encoding a single large polyprotein of about 3000 amino acids. In infected cells, this polyprotein is cleaved at multiple sites by cellular and viral proteases to produce the structural and non-structural (NS) proteins. In the case of HCV, the generation of mature nonstructural proteins (NS2, NS3, NS4A,

25   NS4B, NS5A, and NS5B) is effected by two viral proteases. The first one, as yet poorly characterized, cleaves at the NS2-NS3 junction (henceforth referred to as NS2/3 protease); the second one is a serine protease contained within the N-terminal region of NS3 (NS3 protease) and mediates all the subsequent cleavages downstream of NS3, both in *cis*, at the NS3-NS4A cleavage site, and in *trans*, for the

30   remaining NS4A-NS4B, NS4B-NS5A, NS5A-NS5B sites. The NS4A protein appears to serve multiple functions, acting as a cofactor for the NS3 protease and possibly assisting in the membrane localization of NS3 and other viral replicase components. The complex formation of the NS3 protease with NS4A seems necessary to the processing events, enhancing the proteolytic efficiency at all of the

sites. The NS3 protein also exhibits nucleoside triphosphatase and RNA helicase activities. NS5B is a RNA-dependent RNA polymerase that is involved in the replication of HCV.

5   A general strategy for the development of antiviral agents is to inactivate virally encoded enzymes that are essential for the replication of the virus.

The following is a list of patent applications published in the last few years that disclose HCV NS3 protease inhibitor peptide analogs that are structurally different
10   from the compounds of the present invention:

GB 2,337,262; JP10298151; JP 11126861; JP 11292840; JP 2001-103993;
US 6,159,938; US 6,187,905; WO 97/43310; WO 98/17679; WO 98/22496;
WO 98/46597; WO 98/46630; WO 99/38888; WO 99/50230; WO 99/64442;
15   WO 99/07733; WO 99/07734; WO 00/09543; WO 00/09558; WO 00/20400;
WO 00/59929; WO 00/31129; WO 01/02424; WO 01/07407; WO 01/16357;
WO 01/32691; WO 01/40262; WO 01/58929; WO 01/64678; WO 01/74768;
WO 01/77113; WO 01/81325; WO 02/08187; WO 02/08198; WO 02/08244;
WO 02/08251; WO 02/08256; WO 02/18369; WO 02/60926 and WO 02/79234.

20
One advantage of the present invention is that it provides tripeptide compounds that are inhibitory to the NS3 protease, an enzyme essential for the replication of the hepatitis C virus. Furthermore, the compounds are able to inhibit HCV RNA replication in the replicon cell model.

25
A further advantage of one aspect of the present invention resides in the fact that the compounds specifically inhibit the NS3 protease and do not show significant inhibitory activity against other serine proteases such as human leukocyte elastase (HLE), porcine pancreatic elastase (PPE), or bovine pancreatic chymotrypsin, or
30   cysteine proteases such as human liver cathepsin B (Cat B).

SUMMARY OF THE INVENTION
Included in the scope of the invention is a compound of formula (1):
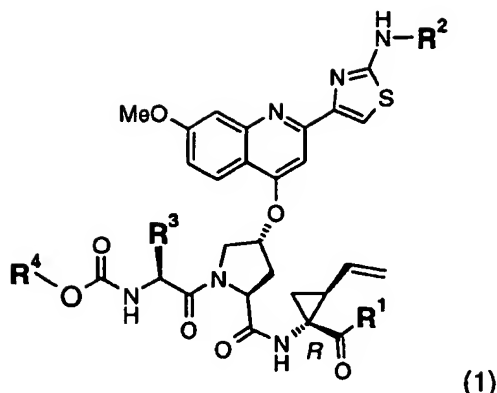
(1)

wherein R^1 is hydroxy or $NHSO_2R^{1A}$ wherein R^{1A} is (C_{1-8})alkyl, (C_{3-7})cycloalkyl or
{(C_{1-6})alkyl-(C_{3-7})cycloalkyl}, which are all optionally substituted from 1 to 3 times
with halo, cyano, nitro, O-(C_{1-6})alkyl, amido, amino or phenyl, or R^{1A} is C_6 or C_{10} aryl

5   which is optionally substituted from 1 to 3 times with halo, cyano, nitro, (C_{1-6})alkyl,
O-(C_{1-6})alkyl, amido, amino or phenyl; R^2 is (C_{4-6})cycloalkyl; R^3 is t-btuyl or (C_{5-6})
cycloalkyl and R^4 is (C_{4-6})cycloalkyl; or a pharmaceutically acceptable salt thereof.

Included within the scope of this invention is a pharmaceutical composition

10   comprising an anti-hepatitis C virally effective amount of a compound of formula I, or
a therapeutically acceptable salt thereof, in admixture with a pharmaceutically
acceptable carrier medium or auxiliary agent.

According to one embodiment, the pharmaceutical composition of this invention

15   further comprises interferon (pegylated or not), or ribavirin, or one or more other
anti-HCV agents, or any combination of the above.

Another important aspect of the invention involves a method of treating a hepatitis C
viral infection in a mammal by administering to the mammal an anti-hepatitis C

20   virally effective amount of a compound of formula I, a pharmaceutically acceptable
salt thereof, or a composition as described above, alone or in combination with one
ore more of: interferon (pegylated or not), or ribavirin, or one or more other anti-HCV
agents, administered together or separately.

25   Another important aspect of the invention involves a method of preventing a
hepatitis C viral infection in a mammal by administering to the mammal an anti-
hepatitis C virally effective amount of a compound of formula I, a pharmaceutically

acceptable salt thereof, or a composition as described above, alone or in combination with one ore more of: interferon (pegylated or not), or ribavirin, or one or more other anti-HCV agent, all of which administered together or separately.

5   Also within the scope of this invention is the use of a compound of formula I, as described herein, for the manufacture of a medicament for the treatment or prevention of hepatitis C viral infection.

The present description refers to a number of documents, the content of which is
10   herein incorporated by reference.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS
Definitions

15   As used herein, the following definitions apply unless otherwise noted:
With reference to the instances where *(R)* or *(S)* is used to designate the absolute configuration of a substituent or asymmetric center of a compound of formula 1, the designation is done in the context of the whole compound and not in the context of the substituent or asymmetric center alone.
20.

The designation "P1, P2, and P3" as used herein refer to the position of the amino acid residues starting from the C-terminus end of the peptide analogs and extending towards the N-terminus (i.e. P1 refers to position 1 from the C-terminus, P2: second position from the C-terminus, etc.) (see Berger A. & Schechter I., Transactions of the
25   Royal Society London series B257, 249-264 (1970)).

As used herein the term "vinyl-ACCA" refers to a compound of formula:
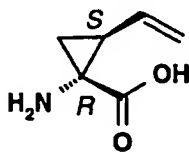
namely, *(1R, 2S)* 1-amino-2-ethenylcyclopropylcarboxylic acid.
30

The term "(C_{1-8})alkyl" as used herein, either alone or in combination with another

substituent, means acyclic straight or branched alkyl substituents containing for 1 to 8 carbon atoms and includes, for example, methyl, ethyl, 2-methylhexyl, 1,1-dimethylhexyl (or t-butyl) and octyl.

5   The term "(C_{3-7})cycloalkyl" as used herein, either alone or in combination with another substituent, means a cycloalkyl substituent containing from 3 to 7 carbon atoms and includes cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl.

The term "{(C_{1-6})alkyl-(C_{3-6})cycloalkyl}" as used herein means a cycloalkyl radical
10   containing from 3 to 6 carbon atoms directly linked to an alkylene radical containing 1 to 6 carbon atoms; for example, cyclopropylmethyl, cyclopentylethyl, cyclohexylmethyl, and cyclohexylethyl. In the instance where R^{1A} is a {(C_{1-6})alkyl-(C_{3-6})cycloalkyl}, this group is attached to the SO_2 group via the (C_{1-6})alkyl (i.e. the alkylene portion).

15

The term "C_6 or C_{10} aryl" as used herein, either alone or in combination with another radical, means either an aromatic monocyclic group containing 6 carbon atoms or an aromatic bicyclic group containing 10 carbon atoms. For example, aryl includes phenyl, 1-naphthyl or 2-naphthyl.

20

The term "$O-(C_{1-6})$ alkyl" as used herein, either alone or in combination with another radical, means the radical $-O-(C_{1-6})$alkyl wherein alkyl is as defined above containing up to six carbon atoms, and includes methoxy, ethoxy, propoxy, 1-methylethoxy, butoxy and 1,1-dimethylethoxy. The latter radical·is known commonly as *tert*-
25   butoxy.

The term "halo" as used herein means a halogen substituent selected from bromo, chloro, fluoro or iodo.

30   The term "pharmaceutically acceptable salt" means a salt of a compound of formula (1) which is, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, generally wat r or oil-soluble or dispersible, and effective for their intended us .

The term includes pharmaceutically-acceptable acid addition salts and pharmaceutically-acceptable base addition salts. Lists of suitable salts are found in, e.g., S.M. Birge et al., J. Pharm. Sci., 1977, 66, pp. 1-19, which is hereby incorporated by reference in its entirety.

5

The term "pharmaceutically-acceptable acid addition salt" means those salts which retain the biological effectiveness and properties of the free bases and which are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, sulfamic acid,

10   nitric acid, phosphoric acid, and the like, and organic acids such as acetic acid, trichloroacetic acid, trifluoroacetic acid, adipic acid, alginic acid, ascorbic acid, aspartic acid, benzenesulfonic acid, benzoic acid, 2-acetoxybenzoic acid, butyric acid, camphoric acid, camphorsulfonic acid, cinnamic acid, citric acid, digluconic acid, ethanesulfonic acid, glutamic acid, glycolic acid, glycerophosphoric acid,

15   hemisulfic acid, heptanoic acid, hexanoic acid, formic acid, fumaric acid, 2-hydroxyethanesulfonic acid (isethionic acid), lactic acid, maleic acid, hydroxymaleic acid, malic acid, malonic acid, mandelic acid, mesitylenesulfonic acid, methanesulfonic acid, naphthalenesulfonic acid, nicotinic acid, 2-naphthalenesulfonic acid, oxalic acid, pamoic acid, pectinic acid, phenylacetic acid,

20   3-phenylpropionic acid, picric acid, pivalic acid, propionic acid, pyruvic acid, salicylic acid, stearic acid, succinic acid, sulfanilic acid, tartaric acid, p-toluenesulfonic acid, undecanoic acid, and the like.

The term "pharmaceutically-acceptable base addition salt" means those salts which

25   retain the biological effectiveness and properties of the free acids and which are not biologically or otherwise undesirable, formed with inorganic bases such as ammonia or hydroxide, carbonate, or bicarbonate of ammonium or a metal cation such as sodium, potassium, lithium, calcium, magnesium, iron, zinc, copper, manganese, aluminum, and the like. Particularly preferred are the ammonium, potassium,

30   sodium, calcium, and magnesium salts. Salts derived from pharmaceutically-acceptable organic nontoxic bases include salts of primary, secondary, and tertiary amines, quaternary amine compounds, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion-exchange resins, such as methylamine, dimethylamine, trimethylamine, ethylamine, diethylamine,

triethylamine, isopropylamine, tripropylamine, tributylamine, ethanolamine, diethanolamine, 2-dimethylaminoethanol, 2-diethylaminoethanol, dicyclohexylamine, lysine, arginine, histidine, caffeine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, methylglucamine, theobromine, purines, piperazine, piperidine, N-

5   ethylpiperidine, tetramethylammonium compounds, tetraethylammonium compounds, pyridine, N,N-dimethylaniline, N-methylpiperidine, N-methylmorpholine, dicyclohexylamine, dibenzylamine, N,N-dibenzylphenethylamine, 1-ephenamine, N,N'-dibenzylethylenediamine, polyamine resins, and the like. Particularly preferred organic nontoxic bases are isopropylamine, diethylamine, ethanolamine,

10   trimethylamine, dicyclohexylamine, choline, and caffeine.

The term "antiviral agent" as used herein means an agent (compound or biological) that is effective to inhibit the formation and/or replication of a virus in a mammal. This includes agents that interfere with either host or viral mechanisms necessary

15   for the formation and/or replication of a virus in a mammal. Antiviral agents include, for example, ribavirin, amantadine, VX-497 (merimepodib, Vertex Pharmaceuticals), VX-498 (Vertex Pharmaceuticals), Levovirin, Viramidine, Ceplene (maxamine), XTL-001 and XTL-002 (XTL Biopharmaceuticals).

20   The term other anti-HCV agent as used herein means those agents that are effective for diminishing or preventing the progression of hepatitis C related symptoms of disease. Such agents can be selected from: anti-viral agents, immunomodulatory agents, inhibitors of HCV NS3 protease, inhibitors of HCV polymerase or inhibitors of another target in the HCV life cycle.

25

The term "immunomodulatory agent" as used herein means those agents (compounds or biologicals) that are effective to enhance or potentiate the immune system response in a mammal. Immunomodulatory agents include, for example, class I interferons (such as α-, β- and omega interferons, tau-interferons,

30   consensus interferons and asialo-interferons), class II interferons (such as γ-interferons) and pegylated interferons.

The term "inhibitor of HCV NS3 protease" as used herein means an agent (compound or biological) that is effective to inhibit the function of HCV NS3 protease

in a mammal. Inhibitors of HCV NS3 protease include, for example, those
compounds described in WO 99/07733, WO 99/07734, WO 00/09558, WO
00/09543, WO 00/59929 or WO 02/060926, the Boehringer Ingelheim clinical
candidate identified as BILN 2061and the Vertex/Eli Lilly pre-development candidate

5   identified as VX-950 or LY-570310. Particularly, compounds # 2, 3, 5, 6, 8, 10, 11,
18, 19, 29, 30, 31, 32, 33, 37, 38, 55, 59, 71, 91, 103, 104, 105, 112, 113, 114, 115,
116, 120, 122, 123, 124, 125, 126 and 127 disclosed in the table of pages 224-226
in WO 02/060926, can be used in combination with the compounds of the present
invention .

10

The term "inhibitor of HCV polymerase" as used herein means an agent (compound
or biological) that is effective to inhibit the function of an HCV polymerase in a
mammal. This includes, for example, inhibitors of HCV NS5B polymerase. Inhibitors
of HCV polymerase include non-nucleosides, for example, those compounds

15   described in:
-        US Application No. 10/198,680, which corresponds to PCT/CA02/01127,
both filed 18 July 2002 (Boehringer Ingelheim),
-        US Application No. 10/198,384, which corresponds to PCT/CA02/01128,
both filed 18 July 2002 (Boehringer Ingelheim),

20   -   US Application No. 10/198,259, which corresponds to PCT/CA02/01129,
both filed 18 July 2002 (Boehringer Ingelheim),
-        WO 02/100846 A1 and WO 02/100851 A2 (both Shire),
-        WO 01/85172 A1 and WO 02/098424 A1 (both GSK),
-        WO 00/06529 and WO 02/06246 A1 (both Merck),

25   -   WO 01/47883 and WO 03/000254 (both Japan Tobacco) and.
-        EP 1 256 628 A2 (Agouron).
Furthermore other inhibitors of HCV polymerase also include nucleoside analogs,
for example, those compounds described in:
-        WO 01/90121 A2 (Idenix),

30   -   WO 02/069903 A2 (Biocryst Pharmaceuticals Inc.), and
-        WO 02/057287 A2 and WO 02/057425 A2 (both Merck/Isis).
Specific examples of inhibitors of an HCV polymerase, include JTK-002, JTK-003
and JTK-109 (Japan Tobacco).

The term "inhibitor of another target in the HCV life cycle" as used herein means an agent (compound or biological) that is effective to inhibit the formation and/or replication of HCV in a mammal other than by inhibiting the function of the HCV NS3 protease. This includes agents that interfere with either host or HCV viral

5   mechanisms necessary for the formation and/or replication of HCV in a mammal. Inhibitors of another target in the HCV life cycle include, for example, agents that inhibit a target selected from a helicase, an HCV NS2/3 protease and an inhibitor of internal ribosomal entry site (IRES). A specific example of inhibitors of another target in the HCV life cycle include ISIS-14803 (ISIS Pharmaceuticals).

10

The term "HIV inhibitor" as used herein means an agents (compound or biological) that is effective to inhibit the formation and/or replication of HIV in a mammal. This includes agents that interfere with either host or viral mechanisms necessary for the formation and/or replication of HIV in a mammal. HIV inhibitors include, for

15   example, nucleosidic inhibitors, non-nucleosidic inhibitors, protease inhibitors, fusion inhibitors and integrase inhibitors.

The term "HAV inhibitor" as used herein means an agent (compound or biological) that is effective to inhibit the formation and/or replication of HAV in a mammal. This

20   includes agents that interfere with either host or viral mechanisms necessary for the formation and/or replication of HAV in a mammal. HAV inhibitors include Hepatitis A vaccines, for example, Havrix® (GlaxoSmithKline), VAQTA® (Merck) and Avaxim® (Aventis Pasteur).

25   The term "HBV inhibitor" as used herein means an agent (compound or biological) that is effective to inhibit the formation and/or replication of HBV in a mammal. This includes agents that interfere with either host or viral mechanisms necessary for the formation and/or replication of HBV in a mammal. HBV inhibitors include, for example, agents that inhibit HBV viral DNA polymerase or HBV vaccines. Specific

30   examples of HBV inhibitors include Lamivudine (Epivir-HBV®), Adefovir Dipivoxil, Entecavir, FTC (Coviracil®), DAPD (DXG), L-FMAU (Clevudine®), AM365 (Amrad), Ldt (Telbivudine), monoval-LdC (Valtorcitabine), ACH-126,443 (L-Fd4C) (Achillion), MCC478 (Eli Lilly), Racivir (RCV), Fluoro-L and D nucleosides, Robustaflavone, ICN 2001-3 (ICN), Bam 205 (Novelos), XTL-001 (XTL), Imino-Sugars (Nonyl-DNJ)

(Synergy), HepBzyme; and immunomodulator products such as: interferon alpha
2b, HE2000 (Hollis-Eden), Theradigm (Epimmune), EHT899 (Enzo Biochem),
Thymosin alpha-1 (Zadaxin®), HBV DNA vaccine (PowderJect), HBV DNA vaccine
(Jefferon Center), HBV antigen (OraGen), BayHep B® (Bayer), Nabi-HB® (Nabi) and
5   Anti-hepatitis B (Cangene); and HBV vaccine products such as the following:
Engerix B, Recombivax HB, GenHevac B, Hepacare, Bio-Hep B, TwinRix, Comvax,
Hexavac.

The term "class I interferon" as used herein means an interferon selected from a
10   group of interferons that all bind to receptor type I. This includes both naturally and
synthetically produced class I interferons. Examples of class I interferons include α-
, β-, omega interferons, tau-interferons, consensus interferons, asialo-interferons.

The term "class II interferon" as used herein means an interferon selected from a
15   group of interferons that all bind to receptor type II. Examples of class II interferons
include γ-interferons.

The pharmaceutical compositions of the invention may contain one or more
additional active agents selected, for example, from antiviral agents,
20   immunomodulatory agents, other inhibitors of HCV NS3 protease, inhibitors of HCV
polymerase, inhibitors of another target in the HCV life cycle, HIV inhibitors, HAV
inhibitors and HBV inhibitors. Examples of such agents are provided in the
Definitions section above.

25   Specific preferred examples of some of these agents are listed below:
   ▪ antiviral agents: ribavirin and amantadine;
   ▪ immunomodulatory agents: class I interferons, class II interferons and pegylated
      interferons;
   ▪ inhibitor of another target in the HCV life cycle that inhibits a target selected
30   from: NS3 helicase, HCV NS2/3 protease or internal ribosome entry site (IRES);
   ▪ HIV inhibitors: nucleosidic inhibitors, non-nucleosidic inhibitors, protease
      inhibitors, fusion inhibitors and integrase inhibitors; or
   ▪ HBV inhibitors: agents that inhibit HBV viral DNA polymerase or is an HBV
      vaccine.

As discussed above, combination therapy is contemplated wherein a compound of formula (1), or a pharmaceutically acceptable salt thereof, is co-administered with at least one additional agent selected from: an antiviral agent, an immunomodulatory agent, another inhibitor of HCV NS3 protease, an inhibitor of HCV polymerase, an

5   inhibitor of another target in the HCV life cycle, an HIV inhibitor, an HAV inhibitor and an HBV inhibitor. Examples of such agents are provided in the Definitions section above. These additional agents may be combined with the compounds of this invention to create a single pharmaceutical dosage form. Alternatively these additional agents may be separately administered to the patient as part of a multiple

10   dosage form, for example, using a kit. Such additional agents may be administered to the patient prior to, concurrently with, or following the administration of wherein a compound of formula (1), or a pharmaceutically acceptable salt thereof.

As used herein, the term "treatment" means the administration of a compound or

15   composition according to the present invention to alleviate or eliminate symptoms of the hepatitis C disease and/or to reduce viral load in a patient.

As used herein, the term "prevention" means the administration of a compound or composition according to the present invention post-exposure of the individual to the

20   virus but before the appearance of symptoms of the disease, and/or prior to the detection of the virus in the blood.

Preferred embodiments

Preferably, compounds of formula 1 as defined above wherein R^1 is hydroxy,

25     $NHSO_2Me$, $NHSO_2cyclopropyl$ or $NHSO_2Ph$. More preferably, R^1 is $NHSO_2$-cyclopropyl or $NHSO_2Ph$. Alternatively, most preferably, R^1 is hydroxy.

Preferably, compounds of formula 1 as defined above wherein R^2 is cyclopentyl or cyclohexyl. Most preferably, R^2 is cyclopentyl.

30

Preferably, R^3 is t-butyl or cyclohexyl. Most preferably, R^3 is t-butyl.

Preferably, compounds of formula 1 as defined above wherein R^4 is cyclobutyl or

cyclopentyl. Most preferably, R^4 is cyclopentyl.

More preferably, a compound of formula 1 as defined above wherein R^1 is hydroxy, R^2 and R^4 each is cyclopentyl and R^3 is t-butyl.

More preferably, a compound of formula 1 wherein R^1 is hydroxy, R^2 is cyclobutyl, R^3 is t-butyl and R^4 is cyclopentyl.

More preferably, a compound of formula 1 wherein R^1 is hydroxy, R^2 is cyclohexyl, R^3 is t-butyl and R^4 is cyclopentyl.

More preferably R^1 is $NHSO_2Ph$, R^2 and R^4 each is cyclopentyl and R^3 t-butyl.

More preferably, a compound of formula 1 wherein R^1 is hydroxy, R^2 is cyclopentyl, R^3 is t-butyl and R^4 is cyclobutyl.

More preferably, a compound of formula 1 wherein R^1 is hydroxy, R^2 is cyclopentyl, R^3 is t-butyl and R^4 is cyclohexyl.

More preferably, a compound of formula 1 wherein R^1 is hydroxy, R^2 and R^4 each is cyclopentyl and R^3 is cyclohexyl.

More preferably, a compound of formula 1 wherein R^1 is hydroxy, R^2, R^3 and R^4 each is cyclopentyl.

According to an alternate embodiment, the pharmaceutical composition of this invention may additionally comprise another anti-HCV agent. Examples of anti-HCV agents include, α- (alpha), β- (beta), δ- (delta), γ- (gamma) or ω- (omega) interferon, ribavirin and amantadine.

According to another alternate embodiment, the pharmaceutical composition of this invention may additionally comprise another inhibitor of HCV NS3 protease.

According to another alternate embodiment, the pharmaceutical composition of this

invention may additionally comprise an inhibitor of HCV polymerase.

According to yet another alternate embodiment, the pharmaceutical composition of this invention may additionally comprise an inhibitor of other targets in the HCV life cycle, including but not limited to, helicase, NS2/3 protease or internal ribosome entry site (IRES).

The pharmaceutical composition of this invention may be administered orally, parenterally or via an implanted reservoir. Oral administration or administration by injection is preferred. The pharmaceutical composition of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. In some cases, the pH of the formulation may be adjusted with pharmaceutically acceptable acids, bases or buffers to enhance the stability of the formulated compound or its delivery form. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, and intralesional injection or infusion techniques.

The pharmaceutical composition may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example Tween 80) and suspending agents.

The pharmaceutical composition of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring and/or coloring agents may be added.

Other suitable vehicles or carriers for the above noted formulations and compositions can be found in standard pharmaceutical texts, e.g. in "Remington's Pharmaceutical Sciences", The Science and Practice of Pharmacy, 19th Ed. Mack Publishing Company, Easton, Penn., (1995).

Dosage levels of between about 0.01 and about 100mg/kg body weight per day, preferably between about 0.1 and about 50mg/kg body weight per day of the protease inhibitor compound described herein are useful in a monotherapy for the prevention and treatment of HCV mediated disease. Typically, the pharmaceutical composition of this invention will be administered from about 1 to about 5 times per day or alternatively, as a continuous infusion. Such administration can be used as a chronic or acute therapy. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. A typical preparation will contain from about 5% to about 95% active compound (*w/w*). Preferably, such preparations contain from about 20% to about 80% active compound.

As the skilled artisan will appreciate, lower or higher doses than those recited above may be required. Specific dosage and treatment regimens for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health status, sex, diet, time of administration, rate of excretion, drug combination, the severity and course of the infection, the patient's disposition to the infection and the judgment of the treating physician. Generally, treatment is initiated with small dosages substantially less than the optimum dose of the peptide. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. In general, the compound is most desirably administered at a concentration level that will generally afford antivirally effective results without causing any harmful or deleterious side effects.

When the composition of this invention comprise a combination of a compound of formula 1 and one or more additional therapeutic or prophylactic agent, both the compound and the additional agent should be present at dosage levels of between about 10 to 100%, and mor preferably between about 10 and 80% of the dosage

normally administered in a monotherapy regimen.

When these compounds or their pharmaceutically acceptable salts are formulated together with a pharmaceutically acceptable carrier, the resulting composition may

5   be administered *in vivo* to mammals, such as man, to inhibit HCV NS3 protease or to treat or prevent HCV virus infection. Such treatment may also be achieved using a compound of this invention in combination with agents which include, but are not limited to: α-, β-, δ-, ω-, tau, or γ-interferon, ribavirin, amantadine; other inhibitors of HCV NS3 protease; inhibitors of HCV polymerase, inhibitors of other targets in the

10   HCV life cycle, which include but not limited to, helicase, NS2/3 protease, or internal ribosome entry site (IRES); or combinations thereof. The additional agents may be combined with compounds of this invention to create a single dosage form. Alternatively these additional agents may be separately administered to a mammal as part of a multiple dosage form.

15

Accordingly, another embodiment of this invention provides a method of inhibiting HCV NS3 protease activity in a mammal by administering a compound of the formula 1.

20   In a preferred embodiment, this method is useful in decreasing the NS3 protease activity of the hepatitis C virus infecting a mammal.

If the pharmaceutical composition comprises only a compound of this invention as the active component, such method may additionally comprise the step of

25   administering to said mammal an agent selected from an immunomodulatory agent, an antiviral agent, a HCV NS3 protease inhibitor, an inhibitor of HCV polymerase or an inhibitor of other targets in the HCV life cycle such as helicase, NS2/3 protease or IRES. Such additional agent may be administered to the mammal prior to, concurrently with, or following the administration of the composition of this invention.

30

A compound of formula 1 set forth herein may also be used as a laboratory reagent. A compound of this invention may also be used to treat or prevent viral contamination of materials and therefore reduce the risk of viral infection of laboratory or medical personnel or patients who come in contact with such materials

(e.g. blood, tissue, surgical instruments and garments, laboratory instruments and garments, and blood collection apparatuses and materials).

A compound of formula 1 set forth herein may also be used as a research reagent.

5   A compound of formula 1 may also be used as positive control to validate surrogate cell-based assays or *in vitro* or *in vivo* viral replication assays.

Further details of the invention are illustrated in the following examples which are understood to be non-limiting with respect to the appended claims.

Methodology

In general, the compounds of formula 1, and intermediates therefore, are prepared

10   by known methods using reaction conditions which are known to be suitable for the reactants. Several such methods are disclosed in WO 00/09543, WO 00/09558 and US Pat. No. 6,323,180, incorporated herein by reference.

Compounds of formula I wherein R^1 is $NHSO_2R^{1A}$ as defined herein are prepared by

15   coupling the corresponding acid of formula I (i.e. R^1 is hydroxy) with an appropriate sulfonamide of formula $R^{1A} SO_2NH_2$ in the presence of a coupling agent under standard conditions. Although several commonly used coupling agents can be employed, TBTU and HATU have been found to be practical. The sulfonamides are available commercially or can be prepared by known methods.
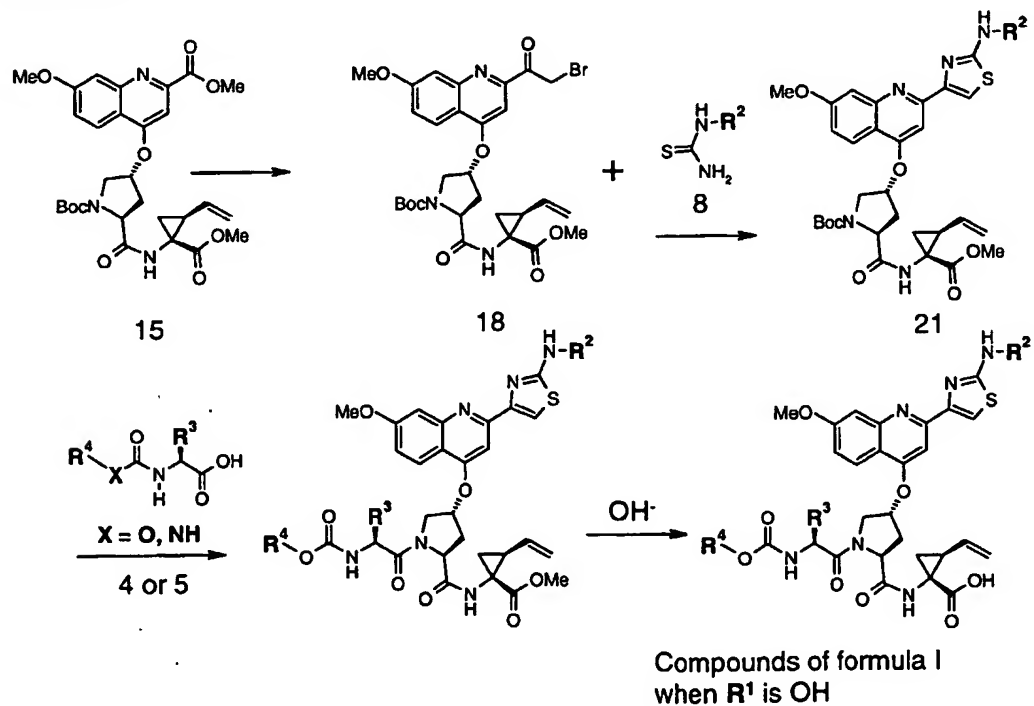
20

The following schemes illustrate two convenient processes using known methods for preparing the compounds of formula 1 when R^1 is OH.

Scheme 1:
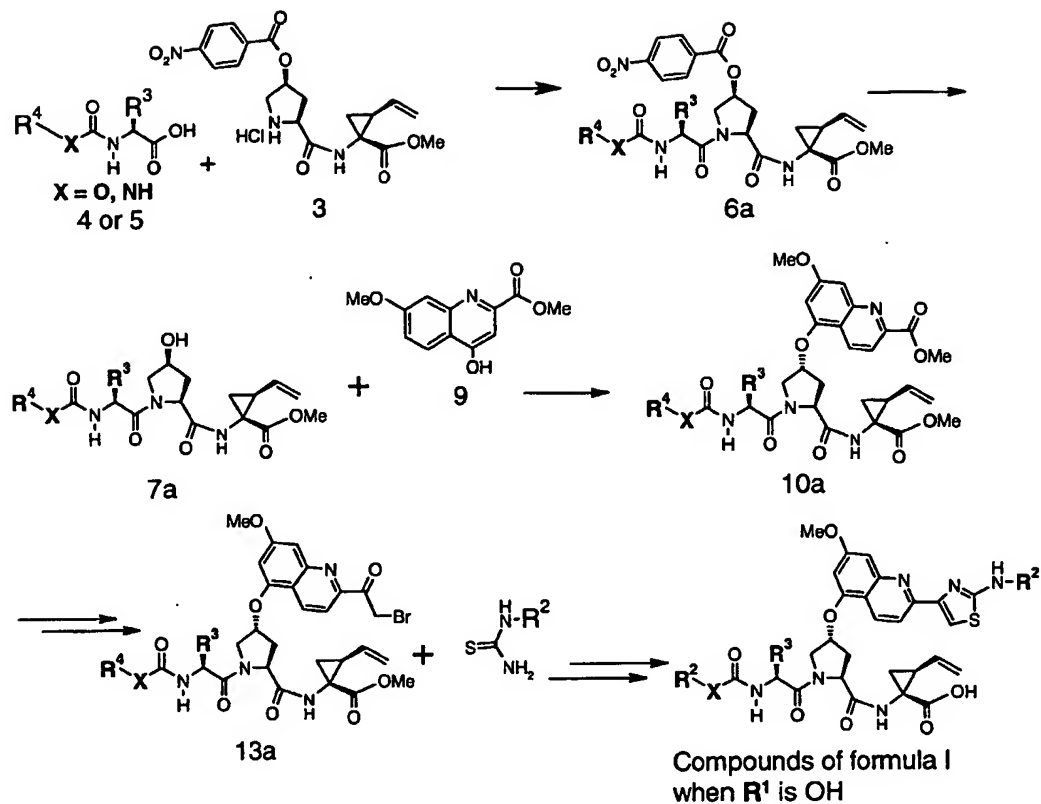
Scheme 2:

EXAMPLES

Temperatures are given in degrees Celsius. Solution percentages express a weight to volume relationship, and solution ratios express a volume to volume relationship,

5   unless stated otherwise. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 400 MHz spectrometer; the chemical shifts (δ) are reported in parts per million. Flash chromatography was carried out on silica gel (SiO_2) according to Still's flash chromatography technique (W.C. Still et al., J. Org. Chem., (1978), 43, 2923).

10

Abbreviations used in the examples include:
DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM: dichloromethane; DIAD: diisopropylazodicarboxylate; DIEA: diisopropylethylamine; DIPEA: diisopropylethyl amine; DMF: N, N-dimethylformamide; DMAP: 4-(dimethylamino) pyridine; EtOAc:

15   ethyl acetate; HATU: [O-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HPLC: high performance liquid chromatography; MS: mass spectrometry (MALDI-TOF: Matrix Assisted Laser Disorption Ionization-Time of Flight, FAB: Fast Atom Bombardment); Me: methyl; MeOH: methanol; Ph: phenyl; R.T.: room temperature (18 to 22°); *tert*-butyl or t-butyl: 1,1-dimethylethyl; Tbg: *tert*-

20   butyl glycine: *tert*-leucine; TBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate; TFA: trifluoroacetic acid; and THF: tetrahydrofuran.

Synthesis of compounds of formula (I):

The dipeptide intermediate **15** (Scheme 2) and 2-carbomethoxy-4-hydroxy-7-

25   methoxyquinoline **9** (Scheme 1) were synthesized according to the methods described in WO 00/09543.


Synthesis of dipeptide 1
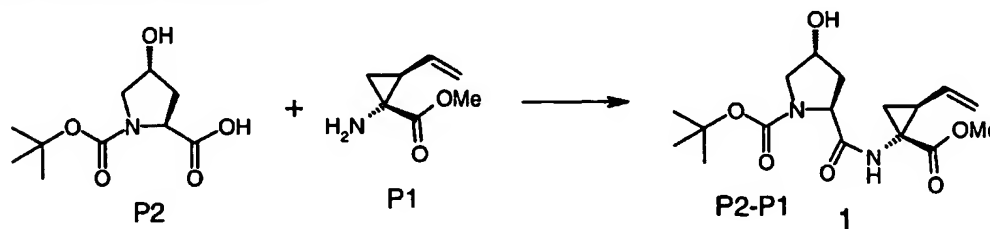
30   A mixture of Boc-hydroxyproline (50.0g, 216mmol), vinyl-ACCA methyl ester

(42.25g, 238mmol, 1.1equiv.), TBTU (76.36g, 238mmol, 1.1equiv.) and DIPEA

(113mL, 649mmol, 3equiv.) in DMF (800mL) was stirred at R.T. under a nitrogen

atmosphere. After 3.5h, the solvent was evaporated and the residue extracted with

EtOAc. The extract was washed with hydrochloric acid (10%), saturated sodium

5   bicarbonate and brine. The organic phase was then dried over magnesium sulfate,

filtered and evaporated to afford an oil. After drying overnight under high vacuum,

dipeptide 1 was obtained as a yellow foam (72.0 g, 94%, purity >95% by HPLC).

Preparation of dipeptide 3

10   1   2   3
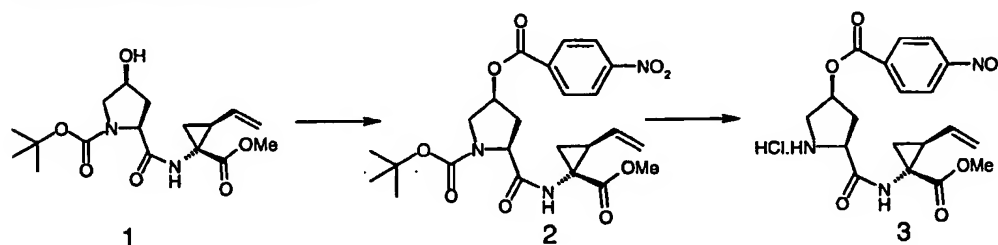
Dipeptide 1 (72.0g, 203mmol), triphenylphosphine (63.94g, 243.8mmol, 1.2equiv.)

and 4-nitrobenzoic acid (41.08g, 245.8mmol, 1.2equiv) were dissolved in dry THF

(1.4L) The stirred solution was cooled to 0°C under a nitrogen atmosphere. Diethyl

azodicarboxylate (38.4mL, 244mmol, 1.2equiv.) was then added dropwise over 45

15   min and the reaction allowed to warm to R.T. After 4h, the solvent was evaporated.

The residue was divided into four portions. Each of these was purified by

chromatography over fine silica gel (10-40μm mesh, column diameter 12cm, column

length 16cm) using a gradient of 2 :1 hexane/EtOAc to 1:1 hexane/EtOAc to pure

EtOAc. In this manner, the Boc-dipeptide ester 2 was obtained as an amorphous

20   white solid after evaporation of the solvents and drying of the residues under high

vacuum at 70°C for 1h (108.1g, quantitative-105%). A solution of 4N hydrogen

chloride in dioxane was added to the Boc-dipeptide ester 2 (108g, 243mmol)

resulting in a colorless solution. The solution was stirred at R.T. for 1h. The solvent

was evaporated and the residue placed under high vacuum for 3h affording the

25   hydrochloride salt of compound 3 as an amorphous solid. The solid was used as

such.

Synthesis of carbamates 4

Preparation of carbamate **4a**:

4a
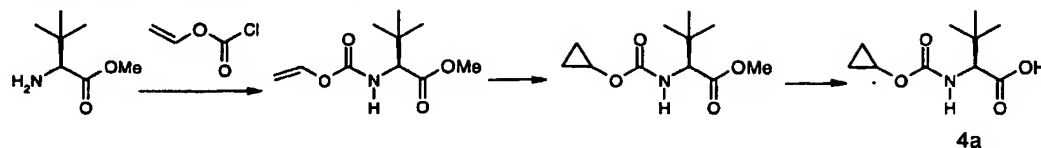
At 0°C, was added to a solution of *tert*-butyl glycine methyl ester (590mg, 3.25mmol)

5 in THF (8mL). vinylchloroformate (0.55mL, 6.47mmol) and triethylamine (1.15mL,
8.25mmol). The temperature was allowed to rise to R.T. The solution was stirred
for 16h. The solution was concentrated and the residue disolved in EtOAc. The
EtOAc solution was washed with a 10% aqueous solution of citric acid (2x), a
saturated aqueous solution of NaHCO$_3$ (2x) and brine, dried and concentrated to

10 yield the corresponding vinyl carbamate (608mg) as a colorless oil. The oil was
dissolved in DCM (4mL), cooled at 0°C, and diiodomethane (0.15mL, 1.86mmol)
and diethylzinc (95µL, 0.93mmol) were added. A white solid appeared at first but
dissolved with time (~ 1h). The suspension/solution was stirred at R.T. for 5h. A
saturated solution of ammonium chloride was added and the solution extracted with

15 EtOAc (2x). The organic extract was dried (MgSO$_4$) and concentrated. The residue
was purified by flash column chromatography. Elution with hexane:EtOAc 95:5 gave
the corresponding methyl ester as a colorless oil (96mg, 90% yield).
A solution the methyl ester (93mg; 0.41mmol) in THF (5mL), MeOH (1mL) and an
aqueous solution of LiOH (45mg ; 1.81mmol) in water (2mL) was stirred for 4h. The

20 solution is dilute with water and extracted with EtOAc (2x). The aqueous solution
was acidified by the addition of 1N HCl, and the acidic solution was extracted with
EtOAc (2x). The combined organic extracts were dried (MgSO4), filtered and
evaporated to obtain the desired carbamate **4a** as a white solid (53mg; 60% yield)

25 Preparation of carbamate **4b**

4b
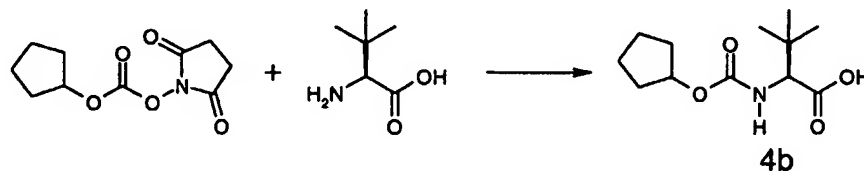
THF (350mL) was added to a flask containing carbonic acid cyclopentyl ester 2,5-
dioxo-pyrrolidin-1-yl ester (9.00g; 39.6mmol) and *tert*-butyl glycine (6.24g;

47.5mmol) resulting in a suspension. Distilled water (100mL) was added with vigorous stirring. A small amount of solid remained undissolved. Triethylamine (16.6mL; 119mmol) was then added resulting in a homogenous solution which was stirred at R.T. After 2.5h, the THF was evaporated and the aqueous residue diluted

5 with water (100mL). The reaction was rendered basic by the addition of 1 N NaOH (25mL - final pH >10). The solution was washed with EtOAc (2 x 200mL) and the aqueous phase acidified with 1 N HCl (ca. 70mL - final pH <2). The turbid solution was extracted with EtOAc (200 + 150mL). The extract was dried (MgSO₄) and evaporated to give carbamate **4b** as a white solid (8.68g).
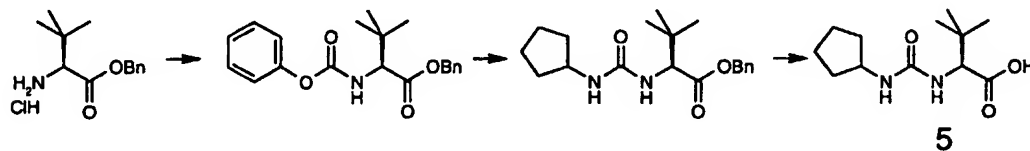
10

Preparation of other carbamates

Using the procedure described above and using appropriate combinations of *tert*-butyl glycine, cyclopentyl glycine, or cyclohexyl glycine and carbonic acid cyclobutyl, cyclopentyl, or cyclohexyl ester 2,5-dioxo-pyrrolidin-1-yl ester, carbamates of the

15 following formulas were prepared:
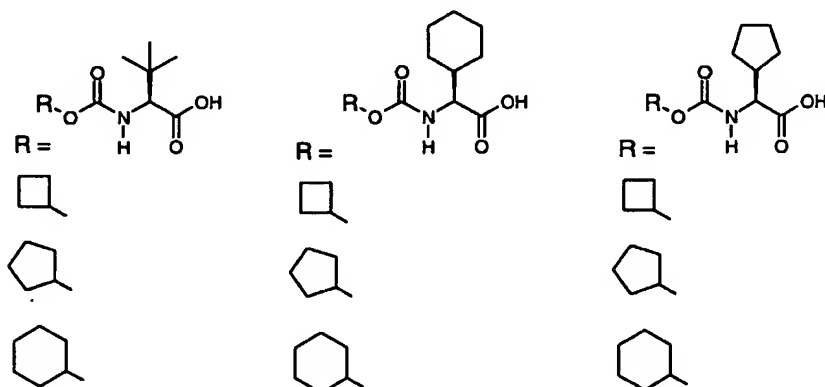
Preparation of Ureas 5

20 A solution of *tert*-butyl glycine benzyl ester hydrochloride salt (2.55g; 9.89mmol) in THF (20mL) and pyridine (2.0mL; 24.73mmol) was cooled to 0°C. Phenyl chloroformate (1.30mL; 10.19mmol) was added dropwise to the cooled solution. The resulting suspension was stirred for 5min at 0°C, then at R.T. for 1.5h. The reaction

mixture was diluted with EtOAc, washed with 10% citric acid (2x) water (2x) saturated $NaHCO_3$ (2x), water (2x) and brine (1x), dried (MgSO4), filtered and evaporated to gain the crude compound as a nearly colorless oil (3.73g ; >100%; assume 9.89mmol). The crude product (1.01g; 2.97mmol) was dissolved in DMSO

5　(6.5mL) and cyclopentylamine was added dropwise. The reaction mixture was stirred at R.T. for 45min. The reaction mixture was diluted with EtOAc. The organic phase was washed with washed with 10% citric acid (2x) water (2x) saturated $NaHCO_3$ (2x), water (2x) and brine (1x), dried (MgSO4), filtered and evaporated to give the crude cyclopentyl urea -Tbg-OBn product as a nearly colorless oil. The

10　crude material was purified by flash column chromatography with silica using hexane:EtOAc 9:1 to remove the less polar impurities and 7:3 to elute the purified product as a thick colourless oil (936mg; 95%). The ester benzyl ester product (936mg; 2.82mmol) was deprotected under a hydrogen filled balloon at R.T. in absolute ethanol (15mL) solution by stirring the solution with 10% Pd/C (93.6mg) for

15　5.5h. The reaction mixture was filtered through a 0.45micron filter and evaporated to dryness to provide urea 5 as a white solid (668.8mg ; 98%)
^{1}H NMR (400 MHz,DMSO-d$_6$): δ 12.39 (s, 1H) , 6.09　(d, J = 7.4 Hz, 1H) , 5.93 (d, J = 9.4 Hz, 1H),　3.90　(d, J = 9.4 Hz, 1H), 3.87-3.77　(m, 1H), 1.84-1.72 (m, 2H), 1.63-1.42 (m, 4H),　1.30-1.19　(m, 2H),　0.89　(s, 9H).

20　M.S.(electrospray) : 241.0 (M-H)-　243.0 (M+H)+ . Reverse Phase HPLC Homogeneity (0.06% TFA; CH_3CN : H_2O) : 99%.

Synthesis of tripeptide 6

4b　+　3　6
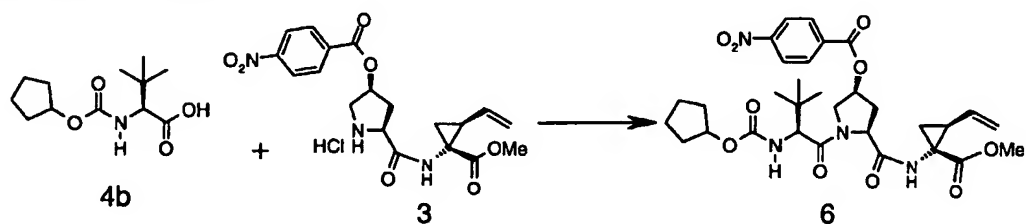
25　Carbamate 4b (6.15g, 22.5mmol) and TBTU (7.72g, 24.7mmol) were suspended in DCM and the suspension was stirred rapidly. DIPEA (3.92mL, 22.5mmol) was added at R.T. and after 10 min, the reaction was nearly homogeneous. A solution of dipeptide 3 (10.39g, 23.6mmol) in anhydrous DCM (100mL) containing DIPEA (4.11mL, 23.62mmol) was then poured into the reaction. The resulting yellow

30　solution was allowed to stir for 14h. The solvent was then evaporated yielding a

yellow syrup which was extracted with EtOAc (300 + 150mL) and washed with 0.05N HCl (2 x 200mL), saturated Na_2CO_3 (300mL) and brine (150mL). The combined extracts were dried over $MgSO_4$ and evaporated to yield the tripeptide **6** as a pale yellow foam (15.68g, quantitiative).

5

Synthesis of tripeptide 7:

6   7
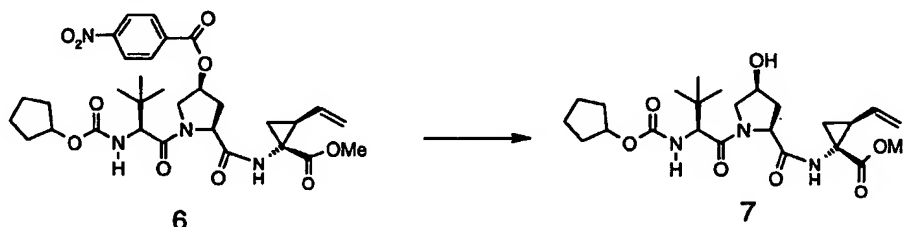
The tripeptide **6** (15.68g) was dissolved in THF (200mL) and water (30mL) was added. The resulting solution was cooled to 0°C and a solution of lithium hydroxide
10   monohydrate (1.18g, 28.12mmol) was added over 3 min with vigorous stirring. After 3h at 0°C, the excess base was neutralized with 1N HCl (final pH ca. 6) and the THF evaporated, resulting in an aqueous suspension (yellow gum). The mixture was extracted with EtOAc (2 x 200mL) and washed with saturated $NaHCO_3$ (2 x 300mL). The combined extracts were dried over $MgSO_4$ and evaporated to yield a pale
15   yellow foam. Flash chromatography of the foam over silica gel using EtOAc as eluent afforded **7** as a white amorphous solid (9.77g, 91%).

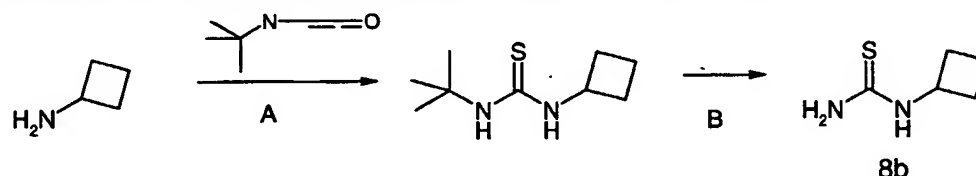**Preparation of Thioureas 8**

Synthesis of thiourea 8a:

20
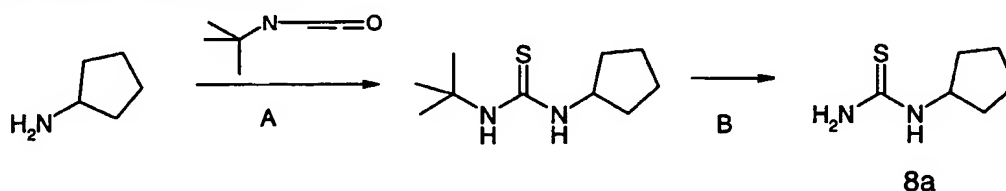
To a solution of *tert*-butyl isothiocyanate (5.0mL; 39.4mmoL) in DCM (200mL) was added cyclopentylamine (4.67mL; 47.3mmoL) followed by DIEA and the reaction mixture was stirred at R.T. for 2h. The mixture was diluted with EtOAc, washed with a 10% aqueous solution of citric acid (2x), saturated $NaHCO_3$ (2x), H_2O (2x) and
25   brine (1x). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to yield N-*tert*-butyl – N'-cyclopentyl thiourea as a white solid (3.70g;

47% yield). The N-*tert*-butyl-N'-cyclopentyl thiourea (3.70g) was dissolved in concentrated HCl (46mL). The dark yellow solution was heated at a gentle reflux. After 40min the reaction mixture was allowed to cool to R.T. and thereafter cooled in ice and rendered basic to pH 9.5 with solid and a saturated aqueous solution of

5   NaHCO₃. The product was extracted into EtOAc (3x). The combined EtOAc extracts were washed with H_2O (2x) and brine (1x). The organic layer was dried (MgSO₄), filtered and concentrated to yield a beige solid (2.46g crude). Trituration of the crude material in hexane/ EtOAc 95 / 5 provided, after filtration, the N-cyclopentythiourea **8a** as a white solid (2.38; 90% yield).

10   ^{1}H NMR (400 MHz,DMSO-d₆): δ7.58 (bs, 1H), 7.19 (bs, 1H), 6.76 (bs, 1H), 4.34 (bs, 1H) , 1.92-1.79 (m,2H), 1.66-1.55 (m, 2H), 1.55-1.30 (m,4H). MS; es⁺ 144.9(M + H)⁺, es⁻: 142.8 (M − H)⁻.
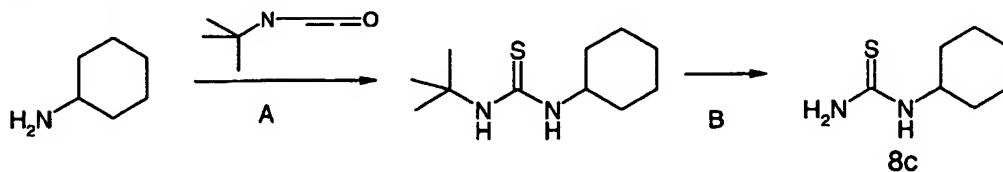

Preparation of thiourea **8b**

15   Using the procedure describe above and using commercially available cyclobutylamine instead of cyclopentylamine yielded thiourea **8b**:
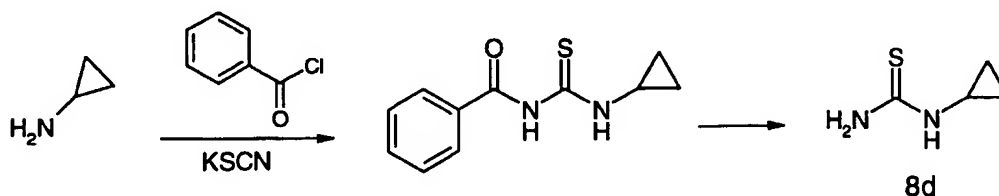

Preparation of thiourea **8c**

20   Using the procedure describe above and using commercially available cyclohexylamine instead of cyclopentylamine yielded thiourea **8c**.

Preparation of thiourea **8d**

To a solution of the KSCN (4.60g; 47.33mmoL) in acetone (35mL), at 0°C, was added dropwise benzoylchloride (5.0mL; 43.03mmoL) . The milky solution was stirred in an ice bath for 1.5h, then, cyclopropylamine (3.2mL; 46.0mmoL) was added dropwise. The reaction mixture was stirred for 1.5h at 0°C, then, more

5　cyclopropylamine (0.50mL, 7.22mmoL) was added and the reaction mixture stirred at R.T. for an additional 30min.　The reaction mixture was poured into ice/H_2O (300mL), stirred for 5min. and the light yellow solid was filtered , washed several times with H_2O and dried to provide N-benzyloxy-N'-cyclopropyl thiourea (6.62g). This thiourea was suspended in a solution of 2N NaOH (50mL) and heated at reflux

10　for 15min. The solution was cooled to R.T., saturated with solid NaCl and extracted with EtOAc (3x). The combined EtOAc extracts were washed with H_2O (2x) and brine (1x), dried (MgSO$_4$), filtered and evaporated to give the crude product as an off-white solid. The solid was triturated in hexane/EtOAc 5/5 to provide the N-cyclopropyl thiourea **8d** as a white crystalline solid (2.5g ; 50% yield).
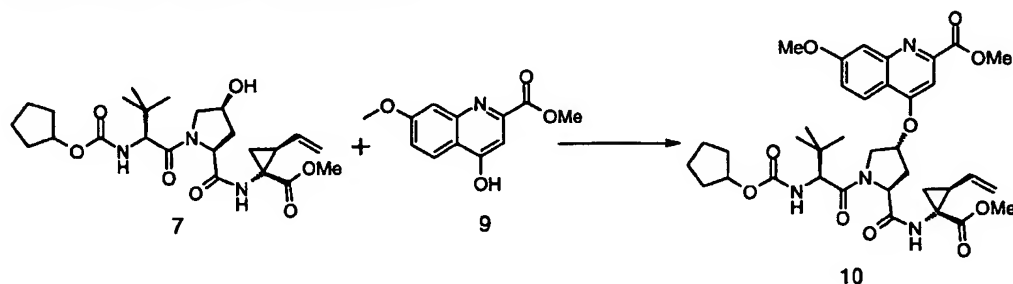
15　^{1}H NMR (400 MHz,DMSO-d$_6$): δ7.92 (bs, 1H), 7.61 (bs, 1H), 7.13 (bs, 1H), 2.39 (bs, 1H) , 0.67-0.63 (m, 2H), 0.51-0.44 (m, 2H).
MS; es$^+$116.9 (M + H)$^+$, es$^-$: 114.8 (M – H)$^-$

EXAMPLE 1

20　Preparation of Compound **100**
Step 1: Synthesis of tripeptide **10**:

To tripeptide 1 (1.0g; 2.09mmol) dissolved in THF (35mL), hydroxyquinoline 9 (729mg; 3.13mmol) and triphenylphosphine (1.1g ; 4.2mmol) were added. The

25　yellow suspension was cooled in an ice bath and DIAD (821μL , 4.2mmol) was added dropwise. The solution was stirred at ice bath temperature for 30 min, and at R.T. for 16h. The solution was evaporated to dryness and the residue was dissolved in EtOAc, washed with a saturated sodium bicarbonate solution (2x), water (2x) and brine (1x), dried (MgSO4), filtered and evaporated to obtain a yellow oil which

precipitated on standing. The crude solid was suspended in DCM and the insoluble

material was filtered off. The solution was concentrated and the residue purified by

flash chromatography in Hexane : EtOAc; 5 : 5 to remove all less polar impurities

and in CHCl₃:EtOAc; 80:20 till all the Ph₃P=O has eluted. The desired compound

5   was eluted with CHCl₃:EtOAc; 65:35 as a white solid (1g; 70% yield).

M.S.(electrospray) : 693.3 (M-H)- 695.4 (M+H)+ 717.4 (M+Na)+ .

Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂0) : 99%.

Step 2 : Selective monohydrolysis of ester 10:

10   10   11
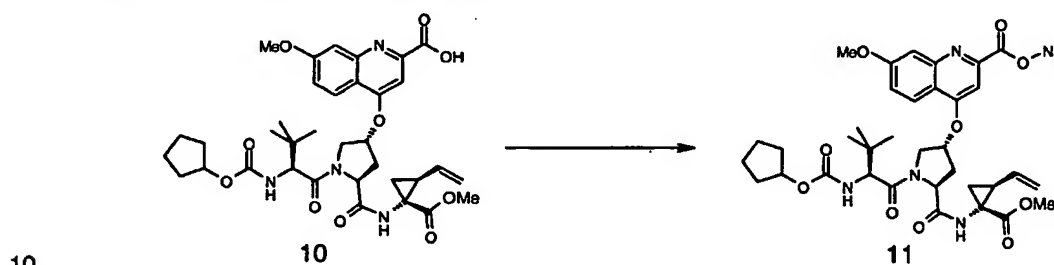
Tripeptide 10 (1g; 1.44mmol) was dissolved in THF (10mL) and MeOH (5mL), water

(5mL) and a 1N NaOH aqueous solution (1.5mL) were added and the solution

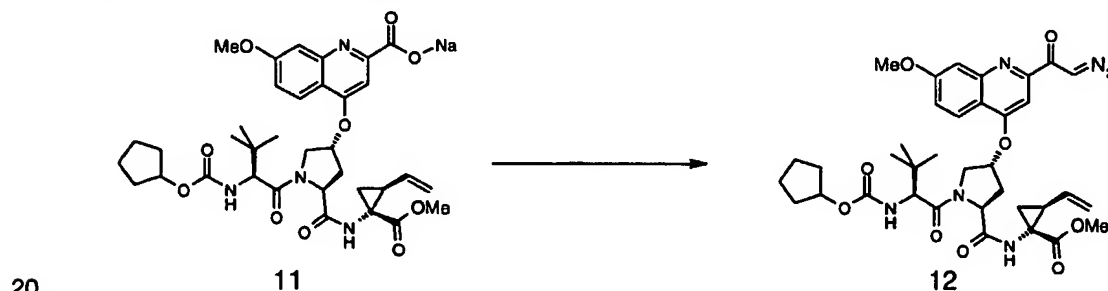stirred at R.T. for 2h. The mixture was evaporated to dryness and then co-

evaporating with MeOH:toluene (1:1; 4x), toluene (2x) and diethyl ether (2x) to

15 obtain the product (water-free) as a white flaky solid (1.04g; 100% yield)

M.S.(electrospray) : 679.3 (M-H)- 681.3 (M+H)+ 703.3 (M+Na)+ .

Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂0) : 95%.

Step 3 : Synthesis of diazoketone 12:

20   11   12

Sodium salt 11 (assume 1.44mmol) was dissolved in THF (16mL), triethylamine

(301µL; 2.16mmol) was added and the solution cooled to 0°C .

Isobutylchloroformate (280µL; 2.16mmol) was added dropwise and the white

suspension was stirred at 0°C for 75min, followed by the addition of a solution of

diazomethane (0.67M in diethyl ether; 13mL; 8.64mmol). The reaction mixture is stirred 1h at 0°C, 45min at R.T. and evaporated to provide a thick suspension. This suspension was dissolved in EtOAc and water. The organic solution was washed with saturated NaHCO₃ (2x), water (2x) and brine (1x), dried (MgSO4), filtered and
5   evaporated to give the diazoketone product as an ivory solid (crude material used for next step; assume 1.44mmol).

M.S.(electrospray) : 703.3 (M-H)- 705.3 (M+H)+ . Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂0) : 91%.

10   Step 4 : Synthesis of bromoketone 13:
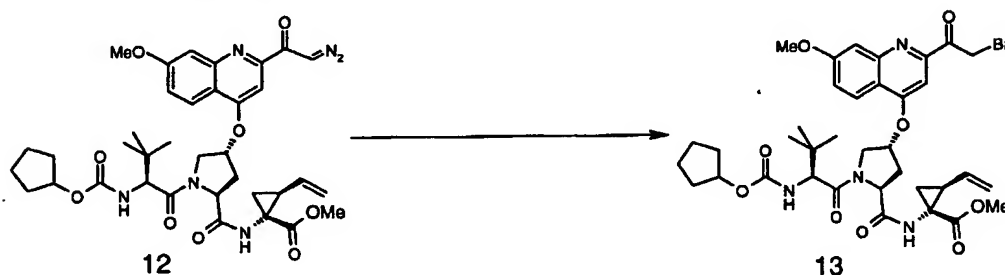
At 0°C, to a solution of diazoketone 12 (1.44mmol) in THF (24mL) was added dropwise an HBr solution (1.0mL) and the mixture was stirred for 1h. The solution was diluted with EtOAc, washed with a saturated NaHCO₃ solution(2x), water (2x)
15   and brine (1x), dry (MgSO4), filtered and evaporated to give the desired bromoketone as an ivory-beige solid (1.1g; assume 1.44mmol).

M.S.(electrospray) : 757.3 (M)   759.3 (M+2)


Step 5 : Synthesis thiazolyl tripeptide 14:
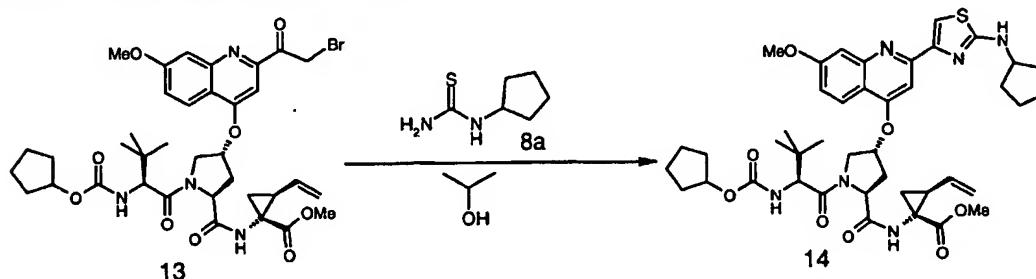
20   α-Bromoketone 13 (0.40mmol) and N-cyclopentylthiourea (68.5mg; 0.48mmol) were dissolved in isopropanol (15mL) and the yellow solution was heated at 70°C for 75min. The solution was allowed to cool to R.T., and evaporated to dryness. The residue was dissolved in EtOAc. The solution was washed with saturated NaHCO₃

(2x), water (2x) and brine (1x), dried (MgSO4), filtered and concentrated to give the product as an orange-brown foam. Flash column chromatography in hexane:EtOAc 7:3 removed less polar impurities and 6 : 4 retrieved the desired compound as a light yellow foam (218mg; 69%).

5   M.S.(electrospray) : 801.4 (M-H)-   803.4 (M+H)+   825 (M+Na)+ .
Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂O) : 99%.

Step 6: Hydrolysis of ester 14:

14   Compound 100
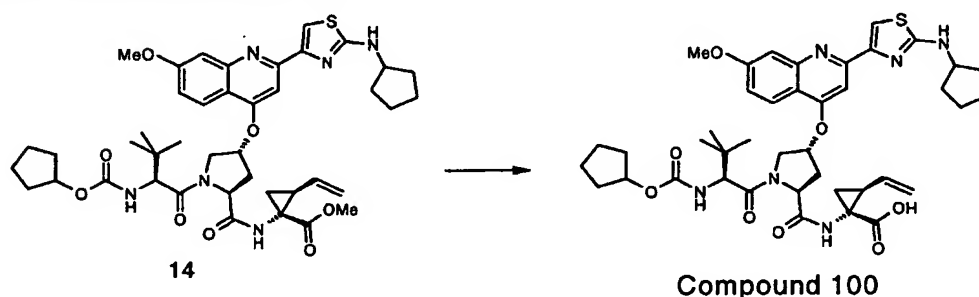
10   A solution of methyl ester 14 (145mg; 0.181mmol) in THF (3mL), MeOH (1.5mL) and an aqueous solution of LiOH (75.8mg; 1.81mmol) in water (1.5mL) was stirred for 18h. The organic solution was concentrated to provide an off-white suspension which was diluted with EtOAc and brine to obtain a total solution. The pH was adjusted to 6 by the addition of 1N HCl and the organic layer was extracted further

15   with EtOAc (2x). The combined organic extracts were washed with water (2x), brine (1x), dried (MgSO4), filtered and evaporated to give the desired compound as a yellow solid (138.2mg; 97% yield).

Conversion to Na Salt:

20   Compound 100 (138.2mg; 0.175mmol) was dissolved in MeOH (30mL) and 1 equivalent 0.01N NaOH (17.5mL) was added. The clear yellow solution was concentrated to remove MeOH and diluted with water, frozen and lyophililzed to obtain the product (Na salt) as a yellow amorphous solid (139mg; theoretical yield : 142mg; MW Na salt : 810.95)

25   M.S.(electrospray) : 787.2 (M-H)-   789.3 (M+H)+   811.3 (M+Na)+ . Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂O) : 98%.
^{1}H NMR (400 MHz,DMSO-d₆): ca, 5:1 mixture of rotamers ;δ☐ 8.14 (bs, 1H) , 8.02 (d, J = 9.2 Hz, 1H) , 7.89 (d, J = 6.7 Hz, 1H), 7.49-7.36 (m, 2H), 7.27 (bs, 1H),

7.06-6.96 (m, 2H), 6.10-5.90 (m, 1H), 5.33 (s, 1H), 5.01 (d, J = 16.8 Hz, 1H), 4.84 (d, J = 10.6 Hz, 1H), 4.79-4.65 (m, 1H), 4.47-4.40 (m, 1H), 4.30 (d, J = 11.5 Hz, 1H),), 4.15 (d, J = 8.8 Hz, 1H), 4.00-3.85 (m, 2H), 3.90 (s, 3H), 2.37-2.26 (m, 1H), 2.15-1.91 (m, 2H), 1.80-1.23 (m, 18H), 0.96 & 0.86 (2x s, 9H).

EXAMPLE 2

Preparation of Compound **101**

Using the same procedure described in example 1 and using N-cyclobutyl thiourea **8b** instead of using N-cyclopentyl thiourea **8a** in step 5 and resulted in compound **101** as theTFA salt:
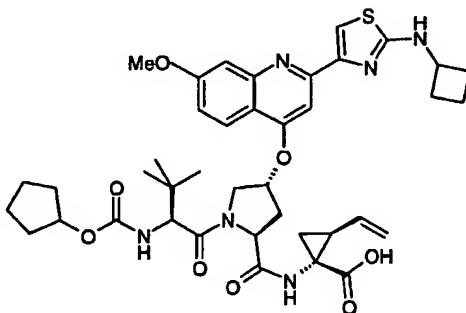
Compound 101

^{1}H NMR (400 MHz,DMSO-d$_6$): ca, 90:10 mixture of rotamers, major isomer description; δ□8.59 (s, 1H), 8.45-8.39 (m, 1H), 8.25 (bs, 1H), 8.20 (d, J = 9.2 Hz, 1H), 7.84 (bs, 1H), 7.74 (s, 1H), 7.32-7.26 (m, 1H), 7.01 (d, J = 8.3 Hz, 1H), 5.78-5.66 (m, 2H), 5.20 (dd, J = 17.0, 1.6 Hz, 1H), 5.09-5.04 (m, 1H), 4.53-4.36 (m, 4H), 4.05-3.92 (m, 2H), 3.97 (s, 3H), 2.63-2.55 (m, 1H), 2.44-2.29 (m, 3H), 2.07-1.95 (m, 3H), 1.79-1.23 (m, 12H), 0.96 (s, 9H).

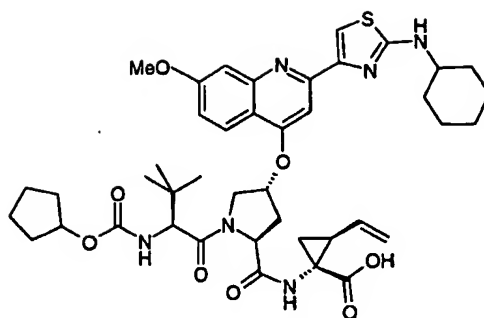M.S.(electrospray) : 773.4 (M-H)- 775.4 (M+H)+ . Reverse Phase HPLC Homogeneity (0.06% TFA; CH$_3$CN : H$_2$0) : 98%

EXAMPLE 3

Preparation of Compound **102**

Using the same procedure described in example 1 and using N-cyclohexyl thiourea **8c** instead of using N-cyclopentyl thiourea **8a** in step 5 and resulted in compound **102** as the TFA salt:

Compound 102

¹H NMR (400 MHz,DMSO-d₆): ca, 90:10 mixture of rotamers, <u>major isomer</u>
<u>description</u>; δ8.61 (s, 1H), 8.26-8.17 (m, 2H), 8.13-8.04 (m, 1H), 7.81 (bs, 1H), 7.73
(bs, 1H), 7.32-7.24 (m, 1H), 7.07 (d, J = 8.2 Hz, 1H), 5.78-5.65 (m, 2H), 5.23-5.15
(m, 1H), 5.09-5.03 (m, 1H), 4.51-4.43 (m, 3H), 4.05-3.77 (m, 3H), 3.97 (s, 3H), 2.64-
2.55 (m, 1H), 2.38-2.27 (m, 1H), 2.05-1.95 (m, 3H), 1.79-1.71 (m, 2H), 1.66-1.19 (m,
16H), 0.96 (s, 9H).

M.S.(electrospray) : 801.4 (M-H)- 803.4 (M+H)+ . Reverse Phase HPLC
Homogeneity (0.06% TFA; CH₃CN : H₂0) : 99%

EXAMPLE 4

<u>Preparation of compound 103</u>

Compound 100   Compound 103
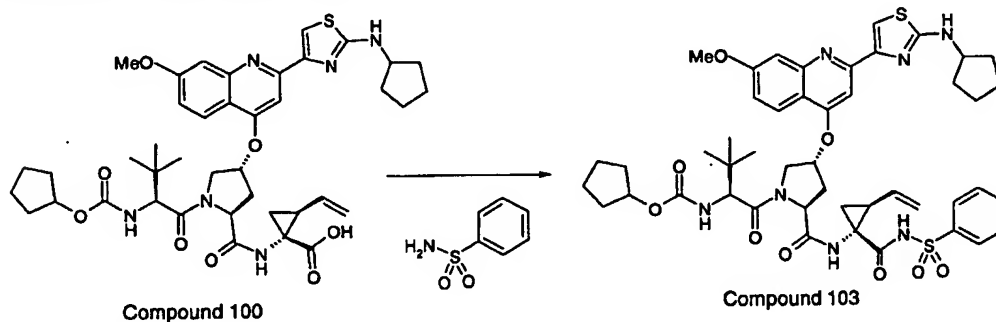
Compound **100** (30mg, 0.038mmol) was combined with HATU (17mg, 0.045mmol)
and dissolved in anhydrous DMF (4mL). The solution was stirred at R.T. before
DIPEA (26µL, 0.15mmol) was added dropwise over *ca.* 1min. The mixture was
stirred for 60min. at R.T. and analyzed by analytical HPLC for the formation of the
activated ester. Following this, a solution of benzenesulfonamide (23mg, 0.15mmol),
DMAP (17mg, 0.14mmol) and DBU (22µL, 0.15mmol) was added in DMF (1mL).
The reaction mixture was stirred 24h at R.T. before being poured into EtOAc (50mL)
and washed with sat. NaHCO₃, and sat. brine solutions. The organic phase was

dried over MgSO₄, filtered and concentrated. The residue was reconstituted in
DMSO and purified by preparative HPLC. Lyophilization gave the final product (17
mg, 48%) as a pale yellow amorphous solid.

5   ^{1}H-NMR (400MHz, DMSO-d6), δ10.89 (s, 1H), 8.84 (s, 1H), 8.20 (d, J = 8.8 Hz, 1H),
8.18-8.08 (m, 2H), 7.89 (d, J = 7.6 Hz, 2H), 7.70 (dd, J = 7.6, 7.6 Hz, 2H), 7.58 (dd,
J = 7.7, 7.7 Hz, 2H), 7.28 (bs, 1H), 7.11 (d, J = 6.6 Hz, 1H), 5.75 (bs, 1H), 5.37-5.25
(m, 1H), 5.12 (d, J = 17 Hz, 1H), 4.92 (d, J = 12 Hz, 1H), 4.58-4.42 (m, 3H), 4.24
(bs, 1H), 4.05 (d, J = 7.8 Hz, 1H), 3.97 (s, 3H), 3.93 (d, J = 7.8 Hz, 1H), 2.69-2.61
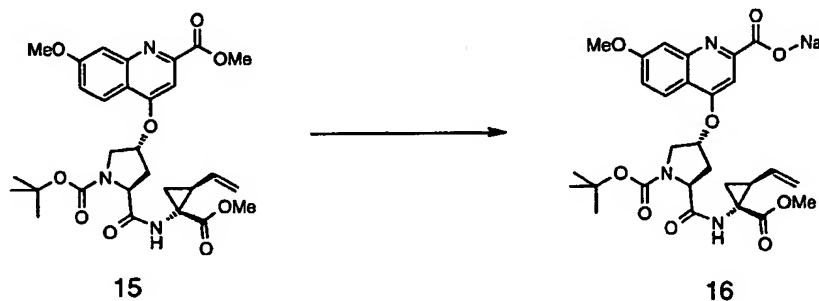(m, 1H), 2.35-2.21 (m, 1H), 2.13-1.98 (m, 3H), 1.78-1.68 (m, 4H), 1.68-1.51 (m, 8H),

10   1.50-1.41 (m, 4H), 1.29-1.22 (m, 1H), 0.99 (s, 9H).
MS (electrospray): 928.5 (M + H)+, and 926.5 (M − H)-.
RP-HPLC: Rt = 7.3 minutes (homogeneity = 99%).

Using the synthetic sequence shown in Scheme 2 the following compounds were

15   prepared:

EXAMPLE 5

Step 1: synthesis of dipeptide **16**:

15   16

20   Dipeptide **15** (4.0g ; 7.02mmol) was dissolved in THF (20mL) and MeOH (10mL),
water (10mL) and a 1N NaOH aqueous solution (1.05 equivalents; 7.4mL) was
added. The solution stirred at R.T. for 2.75h. The mixture was evaporated to
dryness. The residue was diluted with water, frozen and lyophilized to provide
sodium salt **16** as a white amorphous solid (4.28g).

25   M.S.(electrospray) : 554.2 (M-H)- 556.3 (M+H)+   578.2 (M+Na)+ .
Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂0) : 96%.

Step 2: synthesis of dipeptide diazoketone **17**:

16   17
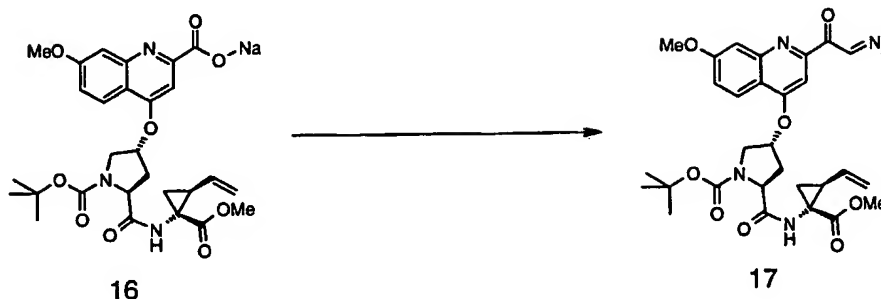
Sodium salt **16** (assume 7.02mmol) was dissolved in THF (78mL); triethylamine (1.37mL; 9.83mmol) was added and the solution cooled to 0°C .

5   Isobutylchloroformate (1.28mL; 9.83mmol) was added dropwise and the white suspension was stirred at 0°C for 2h, , followed by the addition of a solution of diazomethane (0.67M in diethyl ether; 63mL; 42.13mmol). The reaction mixture was stirred 1h at 0°C, 1.25h at R.T. and evaporated to provide a thick suspension. This suspension was dissolved in EtOAc and water. The organic solution was washed

10   with saturated NaHCO₃ (2x), water (2x) and brine (1x), dried (MgSO4), filtered and evaporated to give the diazoketone **17** as an beige solid (crude material used for next step; assume 7.02mmol).

M.S.(electrospray) : 578.2 (M-H)-   580.3 (M+H)+ . Reverse Phase HPLC Homogeneity (0.06% TFA ; CH₃CN : H₂0) : 90%.

15

Step 3: synthesis of dipeptide bromoketone **18**:

17   18
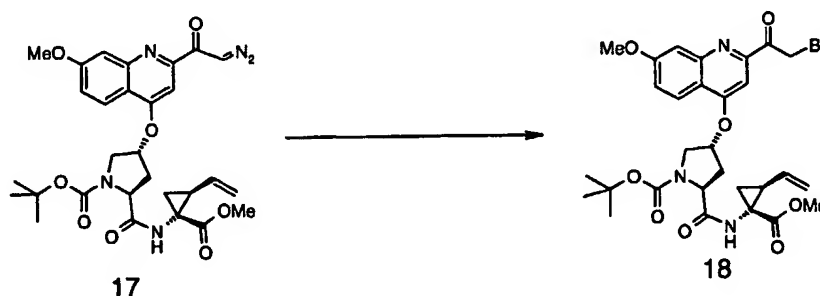
At 0°C, to a solution of diazoketone (assume 1.44mmol) in THF (116mL) was added dropwise a 48% aqueous HBr solution (5.1mL) and the mixture was stirred for 2h.

20   The solution was diluted with EtOAc, washed with a saturated NaHCO₃ solution(2x), water (2x) and brine (1x), dry (MgSO4), filtered and evaporated to give the desired bromoketone as a beige solid (4.25g; 6.72mmol).

M.S.(electrospray) : 632 (M)   634.2 (M+2)

Step 4: synthesis of dipeptide **19**:

18   19
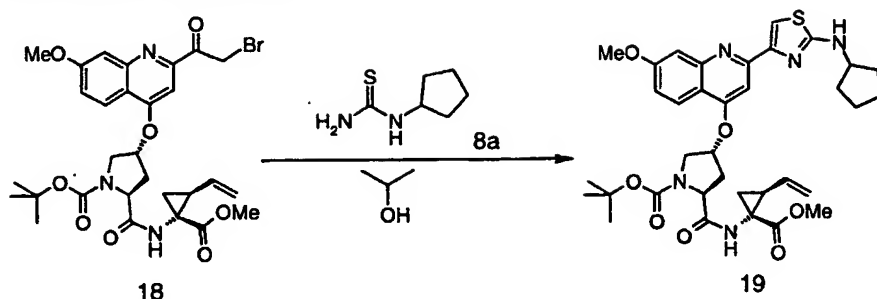
α-Bromoketone **18** (512mg; 0.81mmol) and N-cyclopentylthiourea (128.4mg;

5   0.89mmol) were dissolved in isopropanol (20mL). The resulting yellow solution was
heated at 70°C for 1.5h. The solution was allowed to cool to R.T., and evaporated
to dryness. The residue was diluted with EtOAc. The EtOAc solution was washed
with saturated $NaHCO_3$ (2x), water (2x) and brine (1x), dried (MgSO4), filtered and
concentrated to give the product as an orange-brown foam. Flash column

10 chromatography in hexane:EtOAc 7:3 removed less polar impurities and 6 : 4
retrieved the desired compound as a light yellow solid (411.5mg; 75%).
M.S.(electrospray) : 676.3 (M-H)- 678.3 (M+H)+
Reverse Phase HPLC Homogeneity (0.06% TFA ; CH_3CN : H_2O) : 99%.

15 Step 5: synthesis of tripeptide **20**:

19   20
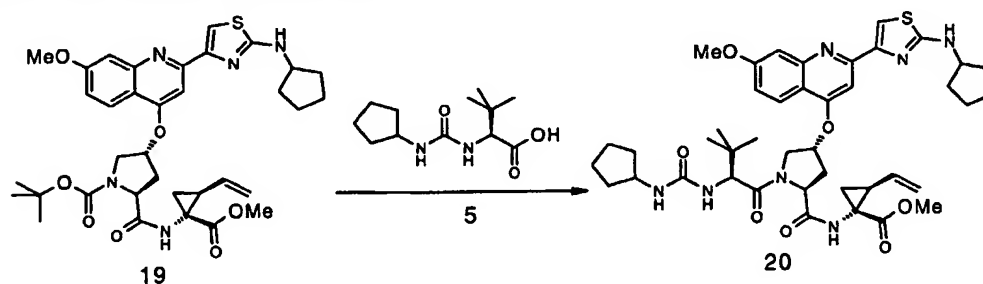
The Boc-dipeptide (32.6g ; 0.048mmol) was dissolved in 4N HCl/dioxane (3mL) and
stirred at R.T. After 2h, the reaction mixture was worked-up by evaporating to
dryness. The HCl salt thus obtained as an off-white solid. The HCl salt was

20 subjected to high vacuum for 30min. To a solution of the HCl salt in DCM (2mL) and
DIEA (33μL ; 0.192mmol), was added urea 5 (13.96mg ; 0.058mmol), followed by
HATU coupling agent (21.90mg ; 0.058mmol). The reaction mixture was stirred at
R.T. for 3h. The reaction mixture was diluted with EtOAc, washed with saturated

NaHCO$_3$ (2x), water (2x) and brine (1x), dried (MgSO4), filtered and evaporated to give the crude product as a thick yellow oil (assume 0.048mmol).

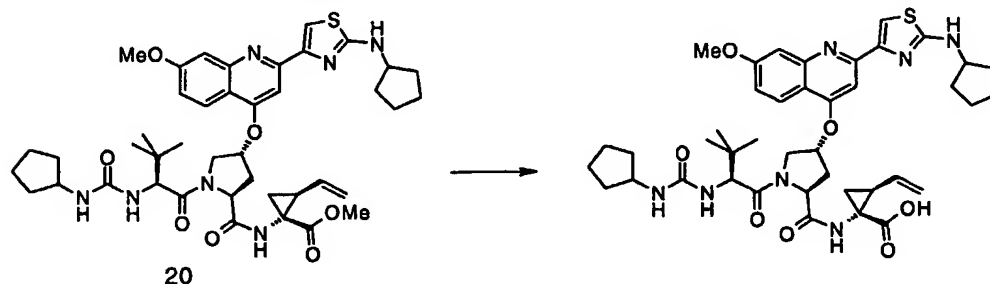M.S. (electrospray) : 800.4 (M-H)- 802.4 (M+H)+ . Reverse Phase HPLC Homogeneity (0.06% TFA ; CH$_3$CN : H$_2$0) : 95%.

5

Step 6: Hydrolysis of tripeptide methyl ester **20**:

20

A solution of methyl ester **20** (77.80mg; 0.097mmol) in THF (2mL), and MeOH (1mL), and an aqueous solution of LiOH (40.7mg; 0.97mmol) in water (1mL), was

10   stirred for 16h. The organic solution was concentrated to provide an off-white suspension. The crude material was purified by preparatory HPLC (YMC Combiscreen ODS-AQ, 50 x20mm ID S-5micron,120A ; λ=220nm) using a linear gradient and 0.06% TFA CH$_3$CN / H$_2$O . The pure fractions were combined, concentrated and converted to the sodium salt.

15

Conversion to Na Salt:

The concentrated fractions were diluted with EtOAc and a few mls of brine, (basified to pH ~13 with 5N NaOH, then, neutralized to pH 5.5 – 6.0 with 1N HCl). The product was extracted with EtOAc (3x), washed with water (2x) and brine (1x),

20   dried (MgSO$_4$), filtered and evaporated to dryness to provide the neutral product as a yellow solid (52.7mg ; 70%). The neutral product (49.4mg ; 0.0627mmol) was dissolved in MeOH (10mL) and 1 equivalent 0.01N NaOH (6.27mL) was added. The clear yellow solution was concentrated to remove MeOH and diluted with water, frozen and lyophililzed to give the product (Na salt) as a yellow amorphous solid

25   (50.8mg; theoretical yield : 50.8mg; MW Na salt : 809.75)

^{1}H NMR (400 MHz,DMSO-d$_6$): ca, 8:1 mixture of rotamers ;δ 8.20 (bs, 1H) , 7.96 (d, J = 8.8 Hz, 1H) , 7.86 (d, J = 5.7 Hz, 1H), 7.51-7.47 (m, 2H), 7.26 (s, 1H), 6.97 (d, J = 8.6 Hz, 1H), 6.19-6.02 (m, 1H), 5.33 (bs, 1H), 4.99 (d, J = 16.8 Hz, 1H), 4.77 (d, J = 10.0 Hz, 1H), 4.52-4.41 (m, 2H), 4.34 (d, J = 11.0 Hz, 1H),), 4.04-3.96

(m, 2H), 3.89 (s, 3H), 3.79-3.68 (m, 1H), 3.68-3.15 (under water peak, 2H), 2.45-2.36 (m, 1H), 2.05-1.92 (m, 2H), 1.82-1.35 (m, 16H), 1.35-1.12 (m, 2H), 0.91 & 0.84 (2x s, 9H).

M.S.(electrospray) : 786.4 (M-H)- 788.3 (M+H)+   810 (M+Na)+ . Reverse Phase
5   HPLC Homogeneity (0.06% TFA ; CH_3CN : H_2O) : 99%.

EXAMPLE 6

Compound 104:

Using the same procedure as described in example 6 and using the cyclobutyl
10   carbamate of *tert*-butyl glycine in step 5 instead of urea 5 and purifying the crude
carboxylic acid after step 6 by preparative HPLC afforded the title compound as a
TFA salt:
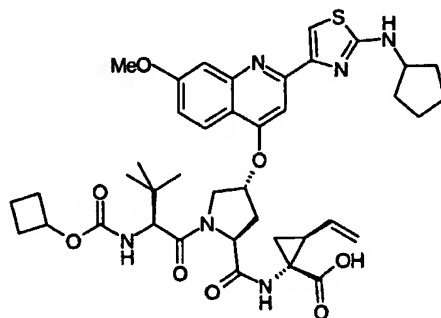
Compound 104

^{1}H NMR (400 MHz,DMSO-d$_6$): ca, 7:1 mixture of rotamers ;δ 8.09 (bs, 1H) , 8.02
15   (d, J = 9.0Hz, 1H) , 7.88 (d, J = 6.5 Hz, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 7.28 (d, J =
2.3 Hz, 1H), 7.16-7.08 (m, 1H), 7.07-7.00 (m, 1H), 6.10-5.95 (m, 1H), 5.32 (bs,
1H), 5.00 (d, J = 17.2 Hz, 1H), 4.82 (d, J = 11.4 Hz, 1H), 4.64-4.52 (m, 1H), 4.48-
4.41 (m, 1H), 4.29 (d, J = 11.7 Hz, 1H),), 4.12 (d, J = 8.6 Hz, 1H), 3.97-3.85 (m,
2H), 3.91 (s, 3H), 2.36-2.27 (m, 1H), 2.18-2.04 (m, 2H), 2.03-1.81 (m, 6H), 1.77-
20   1.43 (m, 9H), 1.41-1.34 (m, 1H), 0.96 & 0.85 (2x s, 9H).

M.S.(electrospray) : 773.3 (M-H)- 775.4 (M+H)+ . Reverse Phase HPLC
Homogeneity (0.06% TFA; CH_3CN : H_2O) : 99%.

EXAMPLE 7

25   Compound 105:

Using the same procedure as described in example 6 and using the cyclohexyl
carbamate of *tert*-butyl glycine in step 5 instead of urea 5 afforded the title

compound as the sodium salt:
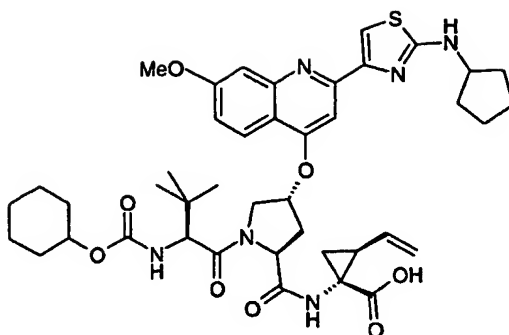
Compound 105

^{1}H NMR (400 MHz,DMSO-d$_6$): ca, 5:1 mixture of rotamers; δ 8.29 (bs, 1H) , 8.02 (d, J = 9.0Hz, 1H) , 7.89 (d, J = 6.5 Hz, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 7.27 (d, J = 2.2 Hz, 1H), 7.05 (d, J = 8.6 Hz, 1H), 7.00 (dd, J = 2.0 , 9.0 Hz, 1H), 5.89 (bs, 1H), 5.34 (s, 1H), 5.08 (d, J = 17.0 Hz, 1H), 4.91 (d, J = 9.4 Hz, 1H), 4.43 (dd, J = 8.4 , 16.8 Hz, 1H), 4.36-4.24 (m, 1H), 4.14 (d, J = 8.6 Hz, 1H),), 4.00-3.86 (m, 3H), 3.89 (s, 3H), 2.35-2.23 (m, 1H), 2.04-1.91 (m, 5H), 1.79-1.41 (m, 10H), 1.39-1.08 (m, 7H), 0.97 & 0.86 (2x s, 9H).

M.S.(electrospray) : 801.4 (M-H)- 803.4 (M+H)+ . Reverse Phase HPLC Homogeneity (0.06% TFA; CH$_3$CN : H$_2$0) : 98%.

EXAMPLE 8

Compound 106:
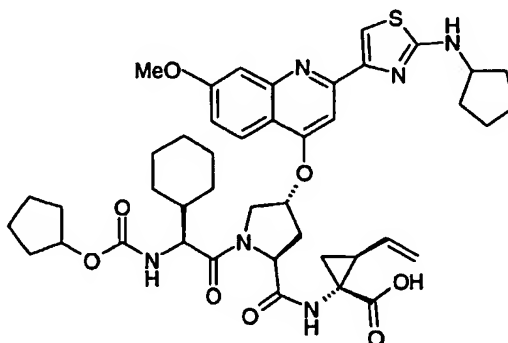
Using the same procedure as described in example 6 and using the cyclopentyl carbamate of cyclohexyl glycine in step 5 instead of urea 5 afforded the title compound as the sodium salt:

^{1}H NMR (400 MHz,DMSO-d$_6$): ca, 1 : 4 mixture of rotamers ;δ 8.22 & 8.04 (2xs, 1H) , 8.00 (d, J = 9.2Hz, 1H) , 7.91 (d, J = 6.5 Hz, 1H), 7.46 (s, 1H), 7.41 (s, 1H), 7.27

(d, J = 2.4 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.01 (dd, J = 2.4 , 9.0 Hz, 1H), 6.09-

5.98 (m, 1H), 5.34 (s, 1H), 4.98 (dd, J = 1.6, 17.4 Hz, 1H), 4.80 (d, J = 11.9 Hz,

1H), 4.73-4.67 (m, 1H), 4.43-4.31 (m, 2H), 4.05-3.95 (m, 2H), 3.95-3.84 (m, 1H),

3.90 (s, 3H), 2.38-2.29 (m, 1H), 2.03-1.92 (m, 2H), 1.83-1.21 (m, 24H), 1.21-0.83

5   (m, 5H)

M.S.(electrospray) : 813.4 (M-H)- 815.4 (M+H)+ . Reverse Phase HPLC

Homogeneity (0.06% TFA; CH_3CN : H_2O) : 99%.

EXAMPLE 9

10   ˙Compound 107:

Using the same procedure as described in example 6 and using the cyclopentyl

carbamate of cyclopentyl glycine instead of urea 5 in step 5, afforded the title

compound which was converted to its corresponding Na salt.

15   **EXAMPLE 10**

NS3-NS4A protease assay

The enzymatic assay used to evaluate the present compound is described in

WO 00/09543 and WO 00/59929.

20   **EXAMPLE 11**

Cell Based HCV RNA Replication Assay

Cell Culture

Huh7 cells that stably maintain a subgenomic HCV replicon were established as

previously described (Lohman et al., 1999. Science **285**: 110-113) and designated

25   as the S22.3 cell-line. S22.3 cells are maintained in Dulbecco's Modified Earle

Medium (DMEM) supplemented with 10% FBS and 1mg/mL neomycin (Standard

Medium). During the assay, DMEM medium supplemented with 10% FBS,

containing 0.5% DMSO and lacking neomycin was used (Assay Medium). 16 hours

prior to compound addition, S22.3 cells are trypsinized and diluted to 50 000 cells/ml

30   in Standard Medium. 200μL (10 000 cells) are distributed into each well of a 96-well

plate. The plate was then incubated at 37° with 5% CO_2 until the next day.

Reagents and Materials:

Pr duct	C mpany	Catal g #	Storage
DMEM	Wisent Inc.	10013CV	4°C
DMSO	Sigma	D-2650	RT
Dulbecco's PBS	Gibco-BRL	14190-136	RT
Fetal Bovine Serum	Bio-Whittaker	14-901F	-20°C/4°C
Neomycin (G418)	Gibco-BRL	10131-027	-20°C/4°C
Trypsin-EDTA	Gibco-BRL	25300-054	-20°C/4°C
96-well plates	Costar	3997	RT
PVDF 0.22µm Filter Unit	Millipore	SLGV025LS	RT
Deep-Well Titer Plate Polypropylene	Beckman	267007	RT

Preparation of Test Compound

10µL of test compound (in 100% DMSO) was added to 2 ml of Assay Medium for a
final DMSO concentration of 0.5% and the solution was sonicated for 15 min and
filtered through a 0.22µM Millipore Filter Unit. 900µl was transfered into row A of a
Polypropylene Deep-Well Titer Plate. Rows B to H, contain 400µL aliquots of Assay
Medium (containing 0.5% DMSO), and are used to prepare serial dilutions (1/2) by
transferring 400µl from row to row (no compound was included in row H).

Application of test compound to cells

Cell culture medium was aspirated from the 96-well plate containing the S22.3 cells.
175µL of assay medium with the appropriate dilution of test compound was
transferred from each well of the compound plate to the corresponding well of the
cell culture plate (row H was used as the "No inhibition control"). The cell culture
plate was incubated at 37° with 5% CO_2 for 72h.

Extraction of Total Cellular RNA

Following the 72h incubation period, the total cellular RNA was extracted from the
S22.3 cells of the 96-well plate using the RNeasy 96 kit (Qiagen®, RNeasy
Handbook. 1999.). Briefly, assay medium was completely removed from cells and
100 µL of RLT buffer (Qiagen®) containing 143 mM β-mercaptoethanol was added

to each well of the 96-well cell-culture plate. The microplate was gently shaken for
20 sec. 100 μL of 70% ethanol was then added to each microplate well, and mixed
by pipetting. The lysate was removed and applied to the wells of a RNeasy 96
(Qiagen®) plate that was placed on top of a Qiagen® Square-Well Block. The

5   RNeasy 96 plate was sealed with tape and the Square-Well Block with the RNeasy
96 plate was loaded into the holder and placed in a rotor bucket of a 4K15C
centrifuge. The sample was centrifuged at 6000 rpm (~5600 x g) for 4 min at room
temperature. The tape was removed from the plate and 0.8 ml of Buffer RW1
(Qiagen® RNeasy 96 kit) was added to each well of the RNeasy 96 plate. The

10   RNeasy 96 plate was sealed with a new piece of tape and centrifuged at 6000 rpm
for 4 min at room temperature. The RNeasy 96 plate was placed on top of another
clean Square-Well Block, the tape removed and 0.8 ml of Buffer RPE (Qiagen®
RNeasy 96 kit) was added to each well of the RNeasy 96 plate. The RNeasy 96
plate was sealed with a new piece of tape and centrifuged at 6000 rpm for 4 min at

15   room temperature. The tape was removed and another 0.8 ml of Buffer RPE
(Qiagen® RNeasy 96 kit) was added to each well of the RNeasy 96 plate. The
RNeasy 96 plate was sealed with a new piece of tape and centrifuged at 6000 rpm
for 10 min at room temperature. Tape was removed, the RNeasy 96 plate was
placed on top of a rack containing 1.2-mL collection microtubes. The RNA was

20   eluted by adding 50 μL of RNase-free water to each well, sealing plate with a new
piece of tape and incubated for 1 min at room temperature. The plate was then
centrifuged at 6000 rpm for 4 min at room temperature. The elution step was
repeated with a second volume of 50 μl RNase-free water. The microtubes with total
cellular RNA are stored at –70°.

25

Quantification of Total Cellular RNA
RNA was quantified on the STORM® system (Molecular Dynamics®) using the
RiboGreen® RNA Quantification Kit (Molecular Probes®). Briefly, the RiboGreen
reagent was diluted 200-fold in TE (10mM Tris-HCl pH =7.5, 1mM EDTA).

30   Generally, 50μL of reagent was diluted in 10mL TE. A Standard Curve of ribosomal
RNA was diluted in TE to 2μg/mL and pre-determined amounts (100, 50, 40, 20, 10,
5, 2 and 0μL) of the ribosomal RNA solution are then transferred in a new 96-well
plate (COSTAR # 3997) and the volume was completed to 100μL with TE.
Generally, column 1 of the 96-well plate was used for the standard curve and the

other wells are used for the RNA samples to be quantified. 10µL of each RNA
sample that was to be quantified, was transferred to the corresponding well of the
96-well plate and 90µL of TE was added. One volume (100µL) of diluted RiboGreen
reagent was added to each well of the 96-well plate and incubated for 2 to 5 minutes
5   at room temperature, protected from light (a 10 µL RNA sample in a 200 uL final
volume generates a 20 X dilution). The fluorescence intensity of each well was
measured on the STORM® system (Molecular Dynamics®). A standard curve was
created on the basis of the known quantities of the ribosomal RNA and the resulting
fluorescent intensities. The RNA concentration in the experimental samples was
10   determined from the standard curve and corrected for the 20X dilution.

Reagents and Materials:

Product	Company	Catalog #	Storage
DEPC	Sigma	D5758	4°C
EDTA	Sigma	E5134	RT
Trizma-Base	Sigma	T8524	RT
Trizma-HCl	Sigma	T7149	RT
Collection Tube Strips	Qiagen	19562	RT
Ribogreen RNA Quantitation Kit	Molecular Probe	R11490	-20°C
Rneasy 96 Kit	Qiagen	74183	RT
Square-Well Blocks	Qiagen	19573	RT

Real-Time RT-PCR

15   The Real-Time RT-PCR was performed on the ABI Prism 7700 Sequence Detection
System using the TaqMan EZ RT-PCR Kit from (Perkin-Elmer Applied
Biosystems®). RT-PCR was optimized for the quantification of the 5' IRES of HCV
RNA by using the Taqman technology (Roche Molecular Diagnostics Systems)
similar to the technique previously described (Martell et al., 1999. J. Clin. Microbiol.
20   37: 327-332). The system exploits the 5'-3' nucleolytic activity of AmpliTaq DNA
polymerase. Briefly, the method utilizes a dual-labeled fluorogenic hybridization
probe (PUTR Probe) that specifically anneals to the template between the PCR
primers (primers 8125 and 7028). The 5' end of the probe contains a fluorescent
reporter (6-carboxyfluorescein [FAM]) and the 3' end contains a fluorescent

quencher (6-carboxytetramethylrhodamine[TAMRA]). The FAM reporter's emission
spectrum was suppressed by the quencher on the intact hybridization probe.
Nuclease degradation of the hybridization probe releases the reporter, resulting in an
increase in fluorescence emission. The ABI Prism 7700 sequence detector

5   measures the increase in fluorescence emission continuously during the PCR
amplification such that the amplified product was directly proportion to the signal.
The amplification plot was analysed early in the reaction at a point that represents
the logarithmic phase of product accumulation. A point representing a defined
detection threshold of the increase in the fluorescent signal associated with the

10   exponential growth of the PCR product for the sequence detector was defined as the
cycle threshold (C_T). C_T values are inversely proportional to the quantity of input
HCV RNA; such that under identical PCR conditions, the larger the starting
concentration of HCV RNA, the lower the C_T. A standard curve was created
automatically by the ABI Prism 7700 detection system by plotting the C_T against

15   each standard dilution of known HCV RNA concentration.

Reference samples for the standard curve are included on each RT-PCR plate. HCV
Replicon RNA was synthesized (by T7 transcription) *in vitro*, purified and quantified
by OD_{260}. Considering that 1μg of this RNA = 2.15 X 10^{11} RNA copies, dilutions are

20   made in order to have 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 or 10^2 genomic RNA copies / 5μL.
Total cellular Huh-7 RNA was also incorporated with each dilution (50ng / 5μL). 5μL
of each reference standard (HCV Replicon + Huh-7 RNA) was combined with 45μL
of Reagent Mix, and used in the Real-Time RT-PCR reaction.

25   The Real-Time RT-PCR reaction was set-up for the experimental samples that were
purified on RNeasy 96 –well plates by combining 5μl of each total cellular RNA
sample with 45μL of Reagent Mix.

Reagents and Materials:

Product	COMPANY	Catalog #	Storage
TaqMan EZ RT-PCR Kit	PE Applied Biosystems	N808-0236	-20°C
MicroAmp Optical Caps	PE Applied Biosystems	N801-0935	RT
MicroAmp Optical 96-Well Reaction Plate	PE Applied Biosystems	N801-0560	RT

Reagent Mix preparation:

Component	V lume for one sample (µL)	Volume f r One Plate (µL) (91 samples + Dead Volume)	Final conc.
Rnase-free water	16.5	1617	
5X TaqMan EZ buffer	10	980	1X
Mn(OAc)$_2$ (25mM)	6	588	3mM
dATP (10mM)	1.5	147	300µM
dCTP (10mM)	1.5	147	300µM
dGTP (10mM)	1.5	147	300µM
dUTP (20mM)	1.5	147	600µM
Forward Primer (10µM)	1	98	200nM
Reverse Primer (10µM)	1	98	200nM
PUTR probe (5µM)	2	196	200nM
rTth DNA polymerase (2.5 U/µL)	2	196	0.1 U/µL
AmpErase UNG (1U/µL)	0.5	49	0.01 U/µL
Total Volume	45	4410	

Forward Primer Sequence (SEQ ID. 1): 5' - ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT - 3'

Reverse Primer Sequence (SEQ ID NO. 2): 5' - TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG - 3'

Note: Those primers amplify a region of 256-nt present within the 5' untranslated region of HCV.

PUTR Probe Sequence (SEQ ID NO. 3): 6FAM - TGG TCT GCG GAA CCG GTG AGT ACA CC - TAMRA

No Template Controls (NTC): On each plate, 4 wells are used as "NTC". For these controls, 5µl of water are added to the well in place of RNA.

Thermal Cycling Conditions:

5
        50°C   2 min
        60°C   30 min
        95°C   5 min

        95°C   15 sec   } for 2 cycles
        60°C   1 min

10
        90°C   15 sec   } for 40 cycles
        60°C   1 min

Following the termination of the RT-PCR reaction the data analysis requires setting of threshold fluorescence signal for the PCR plate and a standard curve was
15 constructed by plotting the Ct value versus RNA copy number used in each reference reaction. The Ct values obtained for the assay samples are used to interpolate an RNA copy number based on the standard curve.

Finally, the RNA copy number was normalized (based on the RiboGreen RNA
20 quantification of the total RNA extracted from the cell culture well) and expressed as genome equivalents / µg of total RNA [ge/µg].

The RNA copy number [g.e./µg] from each well of the cell culture plate was a measure of the amount of replicating HCV RNA in the presence of various
25 concentrations of inhibitor. The % inhibition was calculated with the following equation:

$$100 - [(g.e./\mu g\ inh)/(g.e./\mu g\ ctl) \times 100].$$

A non-linear curve fit with the Hill model was applied to the inhibition-concentration
30 data, and the 50% effective concentration (EC_{50}) was calculated by the use of SAS software (Statistical Software System; SAS Institute, Inc. Cary, N.C.).

When the compounds of this invention were evaluated in the preceding enzymatic and cell based assays, the compounds were found to be highly active. More

- 44 -

specifically, the compounds had IC_{50}'s below 0.1 μM in the NS3-NS4A protease assay, and EC_{50}'s below 0.5 μM in the cell based HCV RNA replication assay.

EXAMPLE 12

5   Specificity assays

The specificity assays used to evaluate the selectivity of this compound are described in WO 00/09543.

When the compounds were evaluated in the specifity assays, the compounds of

10   formula 1 were found to be selective in that they do not show significant inhibition in the Human Leukocyte Elastase and Cathepsin B assays.

EXAMPLE 13

Pharmacokinetic properties

15   The present compounds also show good pharmacokinetic properties such as significant plasma levels in the rat at 1 hour and 2h after an oral dose of 5mg/kg.

More explicitly, the following assay, an *in vivo* oral absorption screen, was used to determine plasma levels of test compounds in a rat after oral administration:

20

Materials and Methods:

1.   Method used to pool compounds ("cassette selection"):

The selection of compounds to be pooled into a "cassette" was based on their structural similarity and physicochemical properties. A solid phase extraction

25   method applicable to all the selected compounds was established. Based on the initial testing where each compound was spiked into rat plasma and run through HPLC or HPLC/MS at a concentration of 0.5 μM, the retention time, ionic mass, and the possible separation among compounds by HPLC and/or HPLC/MS were used as basis for pooling 3-4 compounds into one "cassette".

30   **2.   Oral vehicle and compound preparation:**

Each "cassette" contains 3-4 compounds at 5 or 4mg/kg for each compound. The cass ttes were prepared as an oral suspension in 0.5% aqueous methylcellulose and 0.3% of polyoxyethylene (20) sorbiton monooleate (Tween-80). The dosing volume was 10ml/kg via oral gavage.

3.   D sing and plasma sampling:

Male Sprague Dawley rats were fasted overnight in individual cages, with access to aqueous 10% dextrose. Two rats were dosed with each "cassette". Plasma samples (~1 ml) were collected at 1 and 2h post-dosing from the 2 rats and pooled

5   for extraction and analysis.

**4.   Compound extraction and analysis:**

From each cassette, plasma samples at 1 and 2h, blank plasma, blank plasma spiked with all the compounds at 0.5 µM of each, are extracted by the solid phase extraction method. Samples were analyzed by HPLC and HPLC/MS for comparison

10   purpose. Plasma concentrations are estimated based on the single concentration of 0.5 µM standard.

When assayed in the preceding screen the compounds of examples 1 to 9 of this invention were found to present in significant levels in the plasma at the 1 hour and

15   2 hour intervals following oral administration, averaging blood plasma levels of 1.23 µM and 1.16 µM respectively. This demonstration of significant *in vivo* oral absorption for the compounds of this invention is unexpected, in view of the lower oral absorption generally attributed to this class of peptides. The ready oral absorption renders the compounds useful for treating of HCV infection.

20

The following table lists compounds representative of the invention. Further in keeping with the present disclosure, the compound all had IC_{50}'s below 0.1 µM in the NS3-NS4A protease assay, and EC_{50}'s below 0.5 µM in the cell based HCV RNA replication assay.

TABLE 1

(I)
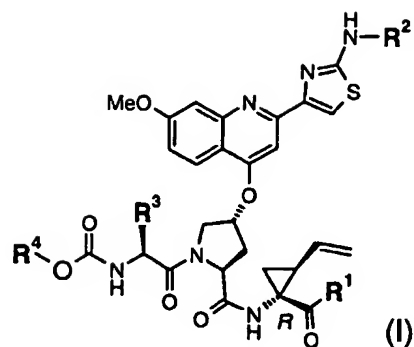
Cpd No.	R¹	R²	R³	R⁴	m/z (MH)⁺
100	OH		*tert*-Butyl		789.3
101	OH		*tert*-Butyl		775.4
102	OH		*tert*-Butyl		803.4
103			*tert*-Butyl		928.5
104	OH		*tert*-Butyl		775.4
105	OH		*tert*-Butyl		803.4
106	OH				815.4
107	OH				801.4

CLAIMS

1.   A compound of formula (I):
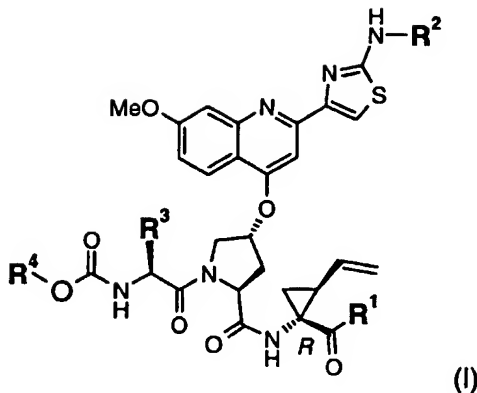
(I)

wherein R^1 is hydroxy or $NHSO_2R^{1A}$ wherein R^{1A} is (C_{1-8})alkyl, (C_{3-7})cycloalkyl or
{(C_{1-6})alkyl-(C_{3-7})cycloalkyl}, which are all optionally substituted from 1 to 3 times
with halo, cyano, nitro, O-(C_{1-6})alkyl, amido, amino or phenyl, or R^{1A} is C_6 or C_{10} aryl
which is optionally substituted from 1 to 3 times with halo, cyano, nitro, (C_{1-6})alkyl,
O-(C_{1-6})alkyl, amido, amino or phenyl; R^2 is (C_{4-6})cycloalkyl; R^3 is t-butyl or (C_{5-6})
cycloalkyl and R^4 is (C_{4-6})cycloalkyl; or a pharmaceutically acceptable salt thereof.

2.   The compound of formula I according to claim 1, wherein R^1 is hydroxy, $NHSO_2Me$,
      $NHSO_2$cyclopropyl or $NHSO_2Ph$.

3.   The compound of formula I according to claim 2, wherein R^1 is $NHSO_2$cyclopropyl
      or $NHSO_2Ph$.

4.   The compound of formula I according to claim 2, wherein R^1 is hydroxy.

5.   The compound of formula I according to any one of claims1 to 4, wherein R^2 is
      cyclopentyl or cyclohexyl.

6.   The compound of formula I according to claim 5, wherein R^2 is cyclopentyl.

7.   The compound of formula I according to any one of claims 1 to 6, wherein R^3 is t-
      butyl or cyclohexyl.

8.   The compound of formula I according to claim 7, wherein R^3 is t-butyl.

9.   The compound of formula I according to any one of claims 1 to 8, wherein R^4 is
      cyclobutyl or cyclopentyl.

10.   The compound of formula I according to claim 9, wherein R^4 is cyclopentyl.

11.   The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2 and R^4
      each is cyclopentyl and R^3 is t-butyl.

12.   The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2 is
      cyclobutyl, R^3 is t-butyl and R^4 is cyclopentyl.

13. The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2 is cyclohexyl, R^3 is t-butyl and R^4 is cyclopentyl.

14. The compound of formula I according to claim 1, wherein R^1 is $NHSO_2Ph$, R^2 and R^4 each is cyclopentyl and R^3 is t-butyl.

15. The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2 is cyclopentyl, R^3 is t-butyl and R^4 is cyclobutyl.

16. The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2 is cyclopentyl, R^3 is t-butyl and R^4 is cyclohexyl.

17. The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2 and R^4 each is cyclopentyl and R^3 is cyclohexyl.

18. The compound of formula I according to claim 1, wherein R^1 is hydroxy, R^2, R^3 and R^4 each is cyclopentyl.

19. A pharmaceutical composition comprising an anti-hepatitis C virally effective amount of a compound of formula 1 according to any one of claim 1 to 18 or a pharmaceutically acceptable salt thereof, in admixture with a pharmaceutically acceptable carrier medium or auxiliary agent.

20. The pharmaceutical composition according to claim 19, further comprising a therapeutically effective amount of one or more other anti-HCV agent.

21. The pharmaceutical composition according to claim 20, wherein said other anti-HCV agent is selected from: α-interferon or pegylated α-interferon.

22. The pharmaceutical composition according to claim 20 or 21, wherein said other anti-HCV agent is ribavirin.

23. The pharmaceutical composition according to claim 20, 21 or 22, wherein said other anti-HCV agent is selected from inhibitors of: helicase, NS2/3 protease and internal ribosome entry site (IRES).

24. A method for the treatment or prevention of a hepatitis C viral infection in a mammal by administering to the mammal an anti-hepatitis C virally effective amount of a compound of formula I according to any one of claims 1 to 18, or a pharmaceutically acceptable salt thereof.

25. A method for the treatment or prevention of a hepatitis C viral infection in a mammal by administering to the mammal an anti-hepatitis C virally effective amount of the composition according any one of claims 19 to 23.

26. A method for the treatment or prevention of a hepatitis C viral infection in a mammal by administering thereto an anti-hepatitis C virally effective amount of a compound

of formula I according to any one of claims 1 to 18, or a therapeutically acceptable salt thereof in combination with one or more other anti-HCV agent.

27.   The method according to claim 26, wherein said other anti-HCV agent is selected from: α-interferon or pegylated α-interferon.

28.   The method according to claim 26 or 27, wherein said other anti-HCV agent is ribavirin.

29.   The method according to claim 26, 27 or 28, wherein said other anti-HCV agent is selected from inhibitors of: helicase, NS2/3 protease and internal ribosome entry site (IRES).

30.   Use of a compound of formula I according to any one of claims 1 to 18 for the manufacture of a medicament for the treatment or prevention of hepatitis C viral infection.

31.   The pharmaceutical composition according to claim 20, 21 or 22, wherein said other anti-HCV agent is an inhibitor of HCV polymerase.

32.   The method according to claim 26, 27 or 28, wherein said other anti-HCV agent is an inhibitor of HCV polymerase.

SEQUENCE LISTING

<110> BOEHRINGER INGELHEIM INTERNATIONAL GmbH

<120> HEPATITIS C INHIBITOR TRI-PEPTIDES

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C07K5/08   A61K38/06   A61P31/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00 09543 A (BOEHRINGER INGELHEIM CA LTD ;GOUDREAU NATHALIE (CA); GHIRO ELISE ()) 24 February 2000 (2000-02-24) cited in the application claims 1,11,17,18,29,33,35,60; table 7 -----	1-32

☐ Further documents are listed in the continuation of box C.   ☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 2003	02/05/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fausti, S

Form PCT/ISA/210 (second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.1

Although claims 24-29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 24-29

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims wer found unsear hable (Continuation f item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 24-29
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

INTERNATIONAL SEARCH REPORT

Inf   Ion on patent family members

Patent document cited in search report		Publication date	Patent family m mber(s)		Publication date
WO 0009543	A	24-02-2000	AU	5273199 A	06-03-2000
			BG	105232 A	30-11-2001
			BR	9913646 A	05-06-2001
			CA	2338946 A1	24-02-2000
			WO	0009543 A2	24-02-2000
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			EE	200100081 A	15-08-2002
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			SK	2062001 A3	08-10-2001
			TR	200100432 T2	21-09-2001
			TR	200200129 T2	21-06-2002
			US	6534523 B1	18-03-2003
			US	6323180 B1	27-11-2001
			US	6268207 B1	31-07-2001
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			US	6329417 B1	11-12-2001
			US	2002016442 A1	07-02-2002
			US	2002037998 A1	28-03-2002